

# **Radiotherapy Supports Tumor Specific Immunity by Acute Inflammation**

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Dissertation

zur  
Erlangung der naturwissenschaftlichen Doktorwürde  
(Dr. sc. nat.)

vorgelegt der  
Mathematisch-naturwissenschaftlichen Fakultät  
der Universität Zürich

von  
**Laura Alba Maria Surace**  
aus  
Italy

## **Promotionskomitee**

Prof. Dr. Maries van den Broek (Leitung der Dissertation & Vorsitz)  
Prof. Dr. Burkhard Becher  
Prof. Dr. Sabine Werner  
Prof. Dr. Martin Pruschy

**Zürich, 2015**

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## Summary

It is now well accepted that the immune system can control tumors. There is a positive correlation between tumor infiltration by effector T cells and survival, and the risk to develop cancer is increased in immunosuppressed patients. Despite the presence of tumor-specific immunity in many cancer patients, complete rejection is rare, presumably due to mechanisms that locally inhibit tumor-specific protective immunity.

Along with surgery and chemotherapy, radiotherapy is an important treatment for cancer. Radiotherapy induces irreversible damage to DNA, thus targeting mainly rapidly dividing cells. Although radiotherapy was considered an immunosuppressive treatment, there is accumulating evidence that it supports local tumor-specific immunity and, in fact, that immune activation might be an integral part of radiotherapy.

In the first part of this study we reported that radiotherapy transiently activated complement within the irradiated tissue resulting in local production of the anaphylatoxins C3a and C5a. Anaphylatoxins were crucial to maturation of radiotherapy-induced maturation of tumor-associated dendritic cells, T cell effector function and clinical efficacy. Our data suggest that radiotherapy induces a local and transient inflammatory response that supports tumor-specific immunity and of which complement is an essential component. In line with this, interference with the inflammatory response by dexamethasone, a corticosteroid often given to patients in the context of radiotherapy, reduced the efficacy of radiotherapy in a preclinical model. We thus propose that the acute inflammatory response that results from radiotherapy actually may contribute to its efficacy.

In the second part of this study, we investigated the impact of radiotherapy on the structure and the function of tumor-associated lymphatic vessels. We observed that radiotherapy did not impact on the structure of lymphatic, but it seemed to enhance lymphatic drainage.

## **Zusammenfassung**

Dass das Immunsystem in der Lage ist, Tumore zu kontrollieren, ist heute eine akzeptierte Tatsache. Das Überleben von Krebspatienten korreliert positiv mit der Menge an Effektor T-Zellen, die in den Tumor einwandern. Ausserdem ist das Risiko an Krebs zu erkranken bei immunsupprimierten Patienten wesentlich höher als bei solchen mit intaktem Immunsystem. Trotz des Vorhandenseins von tumorspezifischer Immunität in vielen Patienten, wird der Tumor oft nicht kontrolliert. Wahrscheinlich gibt es Mechanismen, die die tumorspezifische Immunität lokal unterdrücken.

Zusammen mit Chemotherapie und operativer Entfernung von Tumoren, ist Radiotherapie eine wichtige Therapie für Krebs. Radiotherapie induziert irreversible Schäden in der Erbsubstanz und greift daher hauptsächlich schnell proliferierende Zellen an. Obwohl Radiotherapie lange als immunsuppressiv galt, gibt es nun immer mehr Hinweise dafür, dass Radiotherapie die tumorspezifische Immunität unterstützt und dass die Aktivierung des Immunsystems ein integraler Bestandteil dieser Therapieform ist.

Im ersten Teil dieser Arbeit zeigen wir, dass das Komplement System vorübergehend im bestrahlten Gewebe aktiviert wurde. Das führte zur lokalen Produktion der Anaphylatoxine C3a und C5a. Es zeigte sich, dass Anaphylatoxine für die radiotherapie-induzierte Reifung von Tumorspezifischen dendritischen Zellen, die Funktion von Effektor T-Zellen und die klinische Effizienz von Radiotherapie wichtig waren. Unsere Daten zeigen, dass Radiotherapie eine lokale und vorübergehende Entzündung auslöst, die die tumorspezifische Immunität unterstützt. Das Komplement System ist ein zentraler Bestandteil dieser Entzündungsreaktion. Eine Hemmung der Entzündungsreaktion mit Dexamethason, einem Kortikosteroid, welches im Zusammenhang mit Radiotherapie oft Patienten verabreicht wird, führte in einem präklinischen Modell zum Rückgang der therapeutischen Effizienz der Radiotherapie. Daraus schliessen wir, dass die durch Radiotherapie ausgelöste akute Entzündung wesentlich zur therapeutischen Effizienz beiträgt.

Im zweiten Teil der Arbeit wird der Einfluss von Radiotherapie auf die Struktur und Funktion von Tumor-assoziierten Lymphgefässen untersucht. Wir stellten fest, dass die Struktur durch Radiotherapie nicht beeinflusst wurde, jedoch die Lymphdrainage verstärkt wurde.



# **Introduction**

## **1. The immune system**

### **1.1 Lymphoid organs**

The immune system is composed of organs and vessels that control the production and maturation of immune cells. Primary lymphoid organs (PLOs) including bone marrow and thymus are the main niches for hematopoietic stem cells (HSCs) and are responsible for the generation of leukocytes from progenitor cells. Secondary or peripheral lymphoid organs (SLOs) include spleen, regional lymph nodes (LNs), Peyer's patches, tonsils and mucosa-associated lymphoid tissues (MALT). Here mature lymphocytes are maintained and immune responses are initiated (Randall et al., 2008). During embryogenesis, multi-lineage HSCs arise from specialized vascular endothelial cells in the extraembryonic yolk sac (approx. mouse embryonic day E8.2), in the placenta (day E10) and later in the fetal liver (day E11). HSCs home to fetal bone marrow only shortly before birth (Hirschi, 2012). The thymus, essential for T cells development, derives from the third pharyngeal pouch (day E11) and is situated in the upper anterior thorax, just above the heart. It consists of numerous lobules, clearly differentiated into an outer cortical region and an inner medulla.

SLOs are the sites where an adaptive immune response is initiated. Immune responses against blood-borne antigens are predominantly initiated in the spleen, whereas antigens in tissues reach the local lymph nodes with the interstitial tissue fluid during its passage from the periphery to the thoracic duct. Peyer's patches are the most important and highly organized SLOs of the gut and MALT are diffusely organized aggregates of lymphocytes that protect the gastrointestinal epithelium, together with the tonsils and the respiratory tract.

Whereas PLOs and SLOs develop in the embryo, tertiary lymphoid organs (TLOs) can be formed at any time after birth under conditions of chronic inflammation including autoimmunity and transplant rejection, (chronic) infection or cancer (Aloisi and Pujol-Borrell, 2006). TLOs basic structure is very similar to SLOs (Kratz et al., 1996). The presence of TLOs in autoimmunity and transplant rejection correlates with more severe clinical disease, whereas TLOs seem to have a beneficial role in the context of infections and cancer (Aloisi and Pujol-Borrell, 2006).

### **1.2 General aspects of the immune system**

Traditionally, immunologists have studied the innate and adaptive components of immune defense as two isolated subsystems. Today, these two components are appreciated to act synergistically in protecting the host.

The innate defense comprises a plethora of mechanisms ranging from non-specific barrier function of epithelia to the highly selective recognition of pathogens by germline-encoded receptors. Innate responses are usually initiated by disturbances of homeostasis including pathogens and tissue damage. Granulocytes, monocytes, macrophages, dendritic cells, natural killer (NK) and innate lymphoid cells (ILCs) make up the cellular part of the innate immune system and its most important feature is the ability to respond immediately (Janeway and Medzhitov, 2002).

In contrast, the adaptive immune response is mediated by B and T lymphocytes that recognize unique antigens by their B cell receptor (BCR) and T cell receptor (TCR), respectively. Because the frequency of lymphocytes with a particular specificity is low, a biologically meaningful response can only take place after the relevant lymphocytes have been activated and sufficiently expanded, which usually takes at least a week. The generation of antigen-specific memory is a unique feature of adaptive immunity.

Today this strict distinction between innate and adaptive immunity has become blurred. For example, NK cells show traits that were considered typical for adaptive immunity, such as longevity and recall responses (Sun et al., 2014). In addition,  $\gamma\delta$ T cells, which are classified as innate cells because they recognize nonclassical major histocompatibility complex (MHC) class I molecules and unprocessed proteins, do require recombination-activating gene (RAG)-mediated recombination, possess restricted TCR repertoires and can develop memory responses (Sheridan et al., 2013). Also innate cells that do not require RAG recombination can mount protective recall responses against reinfection, like monocytes (Kleinnijenhuis et al., 2012). The recently discovered and characterized ILC1, ILC2 and ILC3 show functional similarities with  $CD8^+$  T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cells, respectively. ILCs do not undergo antigen-driven clonal selection and expansion, but respond to distinct stimuli. For the moment, they are considered the “blueprint” for the adaptive arm (Eberl et al., 2015), but the question whether these cells have adaptive characteristic remains open. The emerging concept that innate responses control and in fact are a prerequisite for adaptive immunity was introduced by Janeway in 1989 (Janeway, 1989). Many components have been identified as bridging factors between innate and adaptive immunity, directing the field toward an idea of full collaboration between innate and adaptive components (Paul, 2011). Dendritic cells (DCs) are crucial cells linking adaptive and innate immunity and were first discovered by Steinman (Steinman and Cohn, 1973). Dendritic cells develop in the bone marrow and reside as immature DCs in tissues. Upon disturbance, maturation ensues, which results in phenotypic and functional changes enabling the mature DC to initiate T cell responses (Banchereau and Steinman, 1998).

### **1.3 Innate immunity**

The innate immune system has evolved to recognize many different molecular patterns common to pathogens as well as a number of other indicators of cell stress or death. The innate immune system is composed of cellular components that derive from the bone marrow or are maintained by self-renewal in epithelia, where they form the first line of defense against environmental assaults. Moreover, a variety of non-cellular components is part of innate defense, ranging from the barrier function of the stratum corneum or the enzymes secreted in the sweat and saliva to complex systems like anti-microbial peptides (Ganz, 2003), the coagulation cascade and the complement system. These non-cellular components act as a physical blockade and can directly destroy pathogens or send an alarm to other immune cells.

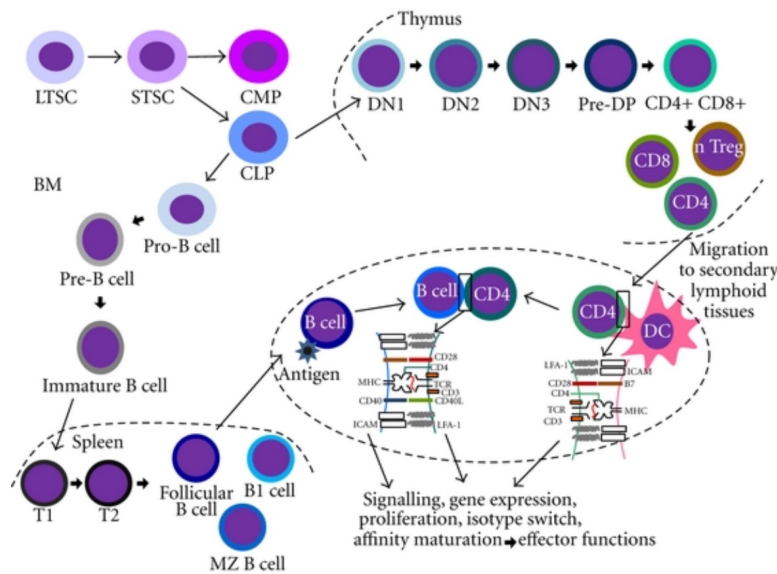
Innate cells recognize pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002) as well as endogenous damages-associated molecular patterns (DAMPs) by pattern-recognition receptors (PRR), which leads to the production of inflammatory mediators and innate host defense. In addition, engagement of PRRs expressed on antigen-presenting cells (APCs), in particular DCs, is a strong maturation stimulus and enables the initiation of adaptive immune response. Several PRRs have been characterized including Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and nucleotide-binding oligomerization domain (Nod)-leucine-rich repeated-containing receptors (NLRs) (Kawai and Akira, 2010). Different PRRs display a PRR-specific subcellular distribution (Barton and Kagan, 2009), which guarantees sensing of most PAMPs (Medzhitov, 2008). In general, the combination of engaged PRRs determines the quality of the following response such that the resulting, integrated immune response is optimally tuned to deal with a particular disturbance (Heredia et al., 2013; Monticelli et al., 2011).

## **1.4 Adaptive immunity**

### **1.4.1 Lymphocyte development**

The main cellular effectors of adaptive immune reactions are B and T lymphocytes. Studies on chicken offered a clear view of separate thymus-dependent (T cells) and bursa-dependent (B cells) lineages of lymphocytes that mediate respectively cellular and humoral response (Cooper et al., 1965). Lymphocyte development starts in the bone marrow (before birth in the fetal liver) from common precursors that commit to B or T cells expressing antigen-specific BCR and TCR, respectively. The antigen specificity of each lymphocyte is determined by the assembly of V (variable), D (diversity) and J (joining) gene segments to generate rearranged V genes encoding the antigen-receptor variable (V) region. This recombination is mediated by RAG1 and RAG2 (Oltz and Osipovich, 2007). The complete antigen receptor requires the rearrangement of two different

genetic loci in order to produce the heavy (H) and the light (L) chain of the BCR or the  $\alpha$  and  $\beta$  chains (or  $\gamma$  and  $\delta$ ) of the TCR. If this rearrangement is successful, the cell proceeds to the next stage of development. The development of both B and T cells depends on stroma cells and it is strictly regulated (Egawa et al., 2001; Janeway, 2001; van Ewijk et al., 1994). Mature lymphocytes circulate through the blood thereby passing through secondary lymphoid organs where they can be exposed to antigens (**Figure 1**).



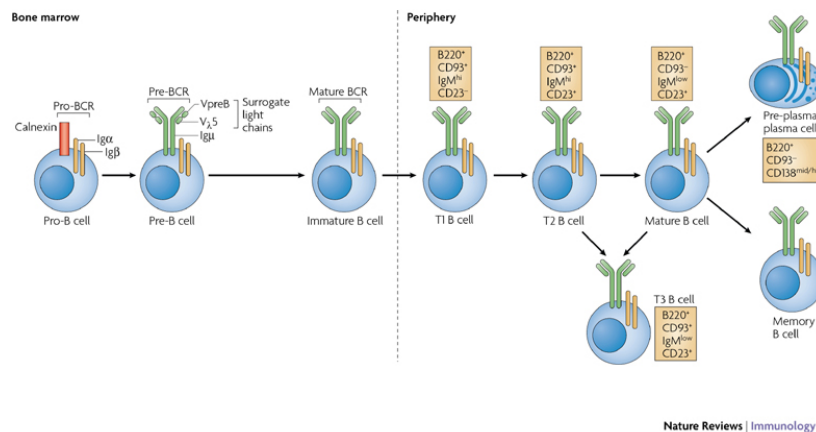
**Figure 1. Lymphocytes development and activation.** Common myeloid and lymphoid progenitors reside in the bone marrow (CMP, CLP). The generation of T cells happens with the migration of progenitors to the thymus where they undergo maturation through different stages till the formation of CD4 and CD8 T cells. B cells develop in the bone marrow and immature B cell can finish their development in the spleen, where they progress to generate marginal zone B cells (MZ B cells), follicular B cells and B1 cells. Mature lymphocytes circulate through SLOs where they encounter antigens directly or on APCs and interact each other (immunological synapses). (Adapted from (Olsen Saraiva Camara et al., 2012)).

### 1.4.2 B cells

B lymphocyte development depends on the nonlymphoid stroma cells in the bone marrow, which interact with the developing B cells through cell-adhesion molecules and production of growth and differentiation factors such as stem cell factor (SCF) and IL-7. Commitment to the B cell lineage is controlled by several transcription factors such as PU.1, IKAROS, E2A, IRF8 and PAX5 (Fuxa and Skok, 2007). The different developmental stages are defined along the functional rearrangement of the immunoglobulin gene segments. Heavy chains are assembled from 4 segments ( $V_H$ , D,  $J_H$  and  $C_H$ ); light chains are assembled from 3 segments ( $V_L$ ,  $J_L$  and  $C_L$ ). In humans, there are 9 different heavy chains types (IgM, IgD, IgG1-4, IgA1 and IgA2, and IgE) and

2 light chain types ( $\kappa$  and  $\lambda$ ). In mice, there are 6 types of heavy chains (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) and 2 light chain types ( $\kappa$  and  $\lambda$ ).

The development of B cells (**Figure 2**) starts with pro-B cells (progenitor cells with limited self-renewal capacity) that rearrange the heavy chain ( $D_H$  to  $J_H$  early and  $V_H$  to  $DJ_H$  late pro-B cells). Productive  $VDJ_H$  joining leads to the expression of the rearranged IgM heavy chain and the formation of pre-B cells. At this stage the IgM heavy chain pairs with surrogate light chains resulting in expression of the pre-B-cell receptor (pre-BCR) on the surface (large pre-B cells). Subsequent to signaling through this receptor, light-chain rearrangement begins forming a complete IgM molecule. At this stage immature B cells undergo a selection process that happens in the bone marrow and subsequently in the SLOs for the elimination of self-reacting cells. Only naïve B cells which complete this process can leave the bone marrow (Janeway, 2001; LeBien and Tedder, 2008).



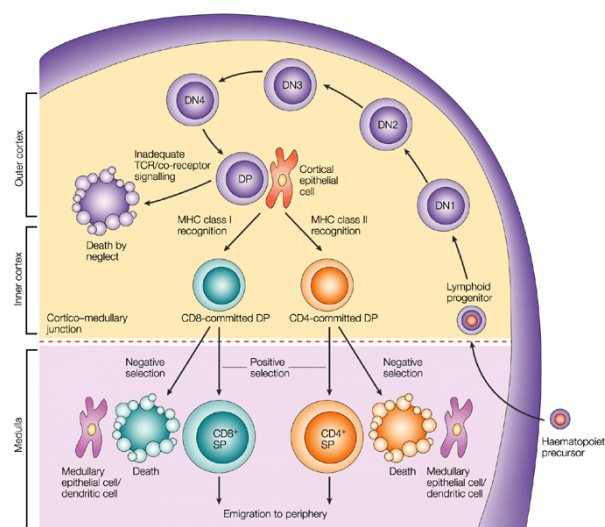
**Figure 2. B-cell development.** In the bone marrow, pro-B cells rearrange the immunoglobulin locus resulting in the generation of the pre-B-cell receptor (pre-BCR). Signaling through this receptor leads to expansion of the precursor, generation of the mature BCR (rearranged heavy- and light-chain genes). These cells are called immature B cells and at this point they undergo a selection process to avoid self-reacting cells. Only cells completing this checkpoint leave the bone marrow as immature B cells, capable of recognizing antigens. They can eventually mature into mature follicular B cells (or marginal-zone B cells). Following an immune response, antigen-specific B cells develop into either plasma (antibody-secreting) cells or memory B cells (Cambier et al., 2007).

B cells reside in the lymphoid follicles of the spleen or the LN where they encounter and respond to antigens, proliferate and differentiate into plasma cells. In this activated form, B cells secrete a free form of the BCR receptors (antibodies or immunoglobulin (Ig)) which have identical binding sites as the ones on the plasma membrane. T-dependent antigens require T cell help for maximal antibody production. When B cells present an antigen on MHC class II in the presence of costimulatory molecules, T cells produce cytokines that lead to immunoglobulin class switching in B cells.

During this process, the heavy chain is changed and the variable region of the heavy chain stays the same. In this way, antibody retains affinity for the same antigens, but can interact with different effector molecules (Gauchat et al., 1990). Immature B cells can recognize T-cell independent antigens such as lipopolysaccharides, eliciting potent response in the absence of MHC class II-restricted T cell-help (Coutinho and Moller, 1975). B cells which do not become plasma cells enter the germinal centers (GC), sites within SLOs where mature B cells undergo clonal expansion, differentiation and somatic hypermutation of  $V_H$  genes and selection for increased affinity of a BCR for its unique epitope (Muramatsu et al., 2000). GCs containing rapidly dividing cells are the main site for high-affinity antibody-secreting plasma cell and memory B cell generation (Jacob et al., 1991). The disruption of this equilibrium regulating activating and inhibitory signals of normal B cell activation and longevity can predispose to pathogenic autoantibody production and autoimmunity (Vinuesa et al., 2009). Besides antibody-dependent defense, B-1 B cells provide an innate protection against bacterial infection (Haas et al., 2005) and marginal zone (MZ) B cells constitute the first line of defense against blood-borne encapsulated bacteria (Pillai et al., 2005).

### 1.4.3 T cells

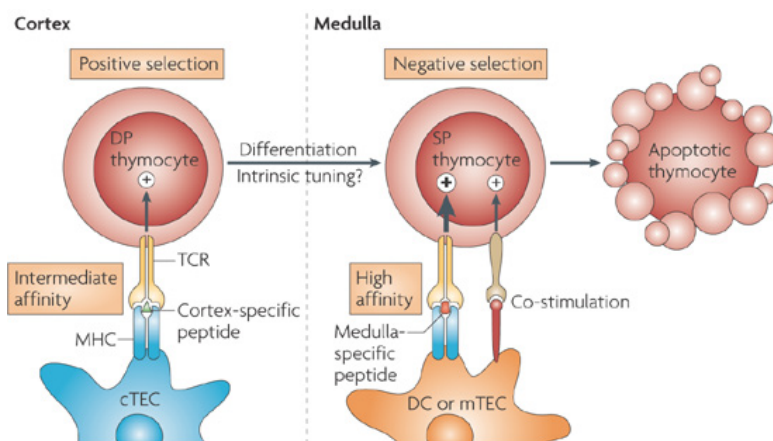
T cells develop in the thymus from common lymphoid progenitor cells (CLPs) (Hedrick, 2008) (**Figure 3**).  $CD4^-CD8^-$  (double negative) CLPs are attracted to the corticomedullary junction by CCL21 and other chemokines. CLPs interact with the adhesion molecules on thymic epithelium, rapidly expand and induce expression of fundamental genes for TCR assembly resulting in commitment to the T cell lineage (Gordon and Manley, 2011).



**Figure 3. Schematic representation of thymic T cell development.** The different stages of T cell maturation from the CLPs to the single positive T cells (SP), happen in different areas of the thymus. The

expression of CD4 or CD8 separates the double positive (DP) and SP cells from the double negative CD4<sup>+</sup>CD8<sup>+</sup> cells (DN) present in the initial stages (Germain, 2002).

During their development, thymocytes rearrange the T cells receptor at the  $\gamma$ ,  $\delta$ , and  $\beta$  loci (MacDonald et al., 2001). In a spatially ordered process, one V, one D and one J segment are randomly spliced together by RAG1 and RAG2. The structures that result from the recombination dictate the amino acid sequence and the binding specificity of the TCR (Oltz and Osipovich, 2007). The successful recombination of *Tcrd* and *Tcrg* concomitant with other signals, promotes  $\gamma\delta$  T cell specification (Lauritsen et al., 2009).  $\alpha\beta$  T cells continue their development and if a stable complex is formed by CD3, TCR $\beta$  and invariant pre-TCR $\alpha$ , they start to travel towards the medulla and to express CD4 and CD8 becoming double positive cells (DP) (von Boehmer, 2005). DP thymocytes that interact with intermediate avidity for self-peptide-MHC complexes presented by cortical thymic epithelial cells (cTECs) undergo positive selection, the surviving DP express self-MHC-restricted TCRs (Klein et al., 2009; Kyewski and Klein, 2006) (**Figure 4**).



**Figure 4. Positive and negative selection.** T cell undergo positive selection in the thymic cortex and it is mediated by cTEC. T cells that survive the process migrate toward the medulla in which they are negatively selected by mTEC and dendritic cells (Klein et al., 2009).

The amount of CD5 on T cells is thought to reflect the signaling intensity of the positively selecting TCR-MHC interaction (Azzam et al., 1998), and the observation that CD5<sup>low</sup> T cell clones are favored in the process of positive selection suggests a selection against TCRs with a too high affinity, maybe to avoid autoimmunity (Nitta et al., 2010). Those T cells that are not positively selected, which are the majority, are eliminated by apoptosis in the thymic cortex (Surh and Sprent, 1994).

Positively selected DP thymocytes commit to the single positive CD4 or CD8 lineage (SP CD4 or SP CD8). SP cells migrate into the medulla where they undergo negative selection or clonal deletion (central tolerance), a process by which TCRs with high affinity for self-antigens are

eliminated, thus reducing the autoreactive T cell pool (**Figure 4**). Medullary thymic epithelial cells (mTECs) express all self proteins, including tissue-restricted antigens (TRAs) (Derbinski et al., 2001) and present them to T cells, which usually results in death of the T cell. This so-called ectopic expression of self proteins depends on the transcription factor Aire and Aire-deficient mice develop spontaneous generalized autoimmunity (Anderson et al., 2002), which underscores the importance of negative selection or central tolerance induction for prevention of autoimmunity. Proteins expressed by mTECs are directly presented to T cells (Hinterberger et al., 2010; Klein et al., 1998; Oukka et al., 1996) or can be handed over to and presented by neighbouring APCs (Koble and Kyewski, 2009). Migratory DCs contribute to clonal deletion because they transport peripheral antigens to the thymic medulla (Baba et al., 2009).

Besides cell death, high-affinity interactions between the TCR and self-peptide-MHC in the thymic medulla can result in development of invariant natural killer (iNKT), CD4<sup>+</sup> regulatory T cells (T<sub>reg</sub> cells) and the precursor of intraepithelial lymphocytes (IELs). This process is called clonal diversion (Cowan et al., 2013; Stritesky and Hogquist, 2012). The specific mechanism how mTECs and DCs manage to elicit clonal diversion is not yet clear, but it seems to be a complex interplay between these two cell types and transcription factors present in the CD4<sup>+</sup> T cells (Lei et al., 2011). Thymocytes with high-affinity TCR can avoid deletion or diversion via secondary gene rearrangement of the TCR $\alpha$  locus, thereby changing the specificity of the TCR in a process known as receptor editing (Holman et al., 2003).

Thus, positive and negative selection favors development of T cells with an adequate affinity to self-MHC and little reactivity to self proteins (Klein et al., 2014). Thymic selection is not 100% accurate and some autoreactive T cells sneak through. Such T cells pose a risk for autoimmunity but at the same time seem instrumental for immunity against cancer. Peripheral tolerance usually prevents activation of such T cells (Yan and Mamula, 2002).

Less than 5% of all thymocytes leave the thymus to differentiate into mature T cells (Starr et al., 2003) that form the long-lived pool of naïve T cells that recirculate within the SLOs. The low self-reactivity that T cells retain after thymic selection is essential for naïve cell survival and enhances the TCR sensitivity for foreign antigens (Klein et al., 2014; Krogsgaard et al., 2007). Recognition of an antigen leads to massive expansion of naïve T cells that goes on for several days, and to the acquisition of a protective effector function. Most effector T cells die within the next weeks, but a small fraction of T cells survive almost indefinitely as memory T cells (Farber et al., 2014; Sallusto and Lanzavecchia, 2001).

TCR $\gamma\delta$  T cells develop in the thymus as well and populate intraepithelial compartments (IEL) of the skin, intestine and genitourinary tract (Asarnow et al., 1988; Sato et al., 1993). They recognize either pathogen-derived antigens or self molecules that reflect a dysregulated status (Janeway et al., 1988). The recognition of these antigens is independent of MHC presentation, but more dependent on conformational shape of intact protein or non-protein compounds (Girardi and Hayday, 2005).



This properties render  $\gamma\delta$  T cells able to participate in the early stages of an immune response in a process named lymphoid stress-surveillance (Hayday, 2009).

NKT cells develop in the thymus and are CD1d-restricted T cells that express an invariant TCR $\alpha$  combined with a limited TCR  $\beta$ -chain repertoire (Godfrey et al., 2004). The resulting TCR recognizes glycolipid antigens presented by CD1d (Bendelac et al., 2001). NKT cells play a role in many different diseases and can both promote and suppress the immune response (Godfrey et al., 2010).

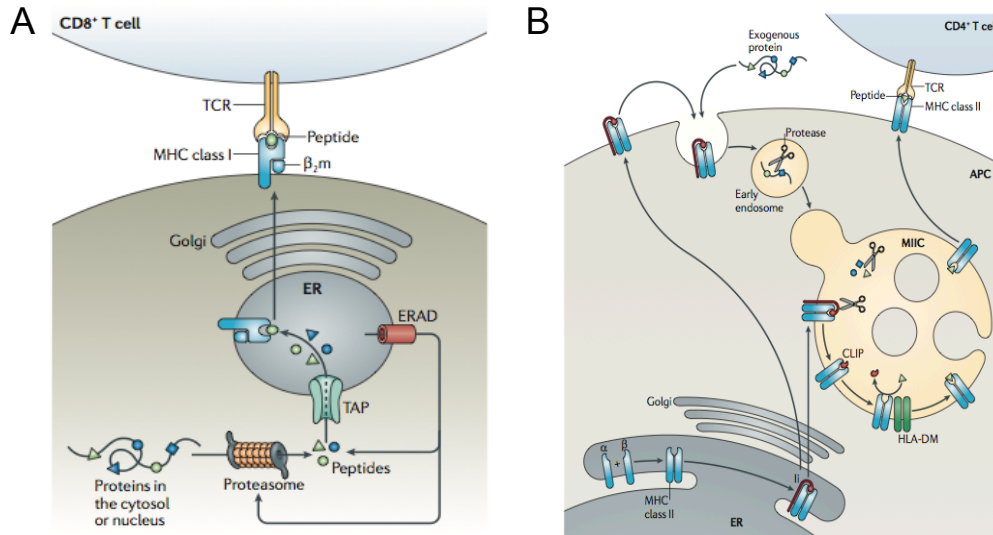
## **2. The immune response**

### **2.1 Antigen presentation and antigen-presenting cells**

MHC molecules are highly polymorphic surface molecules encoded by a large gene family that control major parts of immune responses in vertebrates. The MHC gene family consists of class I, II and III molecules. MHC class I molecules consist of a variable heavy chain that is non-covalently associated with a light conserved  $\beta$ 2-microglobulin molecule, whereas MHC class II molecules consist of two variable chains ( $\alpha$  and  $\beta$  chain) (**Figure 5**). MHC class III molecules comprise proteins that are not involved in antigen presentation such as complement components, heat shock proteins and TNF $\alpha$  (Janeway, 2001). In addition to the highly polymorphic “classical” MHC class I and class II genes, there are other genes linked to the MHC class I region encoding for MHC class 1B molecules. These molecules have different functions, for example, CD1 that is expressed on DCs, thymocytes and monocytes is structurally related to MHC class I and it presents glycolipids in particular to NKT cells (Barral and Brenner, 2007). MHC class I and II molecules binds protein fragments and the peptide/MHC complex can be recognized by T cells through their TCR. MHC class I is expressed by all nucleated cells, whereas the expression of MHC class II is limited to dendritic cells, macrophages and B cells under steady state, although expression can be induced on many cell types under particular conditions such as inflammation. MHC class II expression is tightly controlled by the regulator MHC class II transactivator (CIITA) (Reith et al., 2005).

MHC class I molecules present antigens derived from proteasomal degradation of proteins in the nucleus or in the cytosol. In 1996, Yewdell and colleagues suggested that many presented peptides derive from defective ribosomal products (DRiPs), which are prematurely terminated polypeptides and misfolded proteins that are rapidly degraded and efficiently access the class I pathway. Aminopeptidases trim and destroy most of the peptides and only 0.1% of peptides are translocated into the ER by the transporter associated with antigen presentation (TAP). Peptides of 8-9 amino

acids are loaded on MHC class I molecules by the peptide-loading complex (PLC). Peptide-MHC class I complex is finally released by the PLC and translocated to the cell membrane (Lev et al., 2008; York et al., 2002) (**Figure 5A**).



**Figure 5. The MHC class I and II presentation pathway. A)** MHC class I molecules are situated in the endoplasmic reticulum (ER) and can be loaded with peptides coming from proteins degraded by proteasomes. The transported associated with antigen presentation (TAP) translocates peptides into the ER lumen and when MHC class I are in complex with the antigens, they are transported to the plasma membrane via Golgi and present the antigen to the CD8<sup>+</sup> T cells. **B)** MHC class II molecules are assembled in the ER and then transported via Golgi to the MHC class II compartment (MIIC). The invariant chain (Ii) is degraded in these vesicles along with endocytosed proteins. MHC class II molecules left with the MHC class II associated Ii peptide (CLIP) can be loaded only with high affinity peptides with the help of the chaperon H2-DM. At the end of the process they are transported to the plasma membrane and present the antigen to CD4<sup>+</sup> T cells (adapted from (Neeffjes et al., 2011)).

Specific subsets of APCs can also present exogenous antigens on MHC class I in a process called cross-presentation (Kurts et al., 2010). It has been recently shown that autophagy (a catabolic process by which the cell eliminates unnecessary or dysfunctional components by lysosomal degradation) can result in loading of peptides derived from endogenous proteins on MHC class II (Munz, 2009).

MHC class II subunits are associated to the invariant chain and then transported to late endosomes, also called MHC class II compartment (MIIC). In this compartment, the invariant chain is digested and a peptide is left in the binding groove of the MHC class II heterodimer. The chaperone H2-DM is needed to exchange the peptide in the binding groove with high affinity peptides. MHC class II molecules in complex with peptides are then transported to the plasma membrane to present the

peptide to the CD4<sup>+</sup> T cells (Neefjes et al., 2011) (**Figure 5B**). CD8<sup>+</sup> T cells interact with MHC class I and CD4<sup>+</sup> T cells with MHC class II molecules.

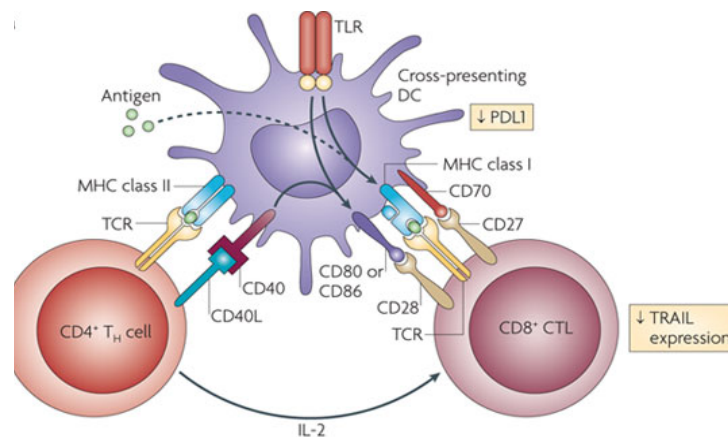
There are many different APCs that can trigger T cells, including B cells, macrophages, endothelial cells and DCs, but DCs are the quintessential professional APCs (Steinman and Cohn, 1973). DCs were first identified by Steinman and Cohn as cells with dendrites, which gave the cells their name (Steinman and Cohn, 1973). DCs originate from bone marrow precursors that seed different tissues via the blood and locally differentiate into specialized sentinels. All DCs subsets are equipped with different PRRs, including TLRs, on the plasma membrane or in the cytosol. Ligation of TLRs leads to DC maturation, which results in the production of soluble factors and endows DCs with the capacity to activate naïve T cells during cognate interaction (Akira et al., 2006; Blander and Medzhitov, 2006; Sporri and Reis e Sousa, 2005). The efficiency of dendritic cells as antigen presenting cells depends on several important characteristics. Upon maturation antigen processing function are increased (Delamarre et al., 2003; West et al., 2004). DCs express high levels of MHC class II (Schuler and Steinman, 1985) and have decreased proteolytic capacity compared to that of other phagocytes, which results in efficient cross-presentation (Delamarre et al., 2005). Moreover, DC phagosomes are characterized by the presence of MHC class I loading ER-resident proteins (Guermónprez et al., 2003; Houde et al., 2003).

DCs can be classified in two main categories: plasmacytoid DCs (pDCs) and conventional DC (cDCs) (Turley et al., 2010). The development of pDCs is controlled by the transcription factor E2-2. pDCs produce type I interferons and IL-12 upon stimulation (Swiecki and Colonna, 2010). Conventional DCs can be subdivided in two categories based on their development: cDC1, controlled by BATF3 and cDC2 by IRF4 (Guilliams et al., 2014). In general, conventional DCs are identified by the surface markers CD11c<sup>+</sup> MHCII<sup>+</sup>, but they can express several other markers based on their function. Recently, the chemokine receptor Xcr1 has been identified as a specific marker for the BATF3-dependent CD8α<sup>+</sup> T cells (Bachem et al., 2012; Dalod et al., 2014). Xcr1<sup>+</sup> DCs have been identified as the most efficient in presenting antigens on cross-presentation (Joffre et al., 2012). However there are some features that could explain the superior cross-presentation capacity: Xcr1<sup>+</sup> DC maintain a less acidic pH in endosomes and phagosomes, favoring cross-presentation from early endocytic vesicles (Savina et al., 2009); they are more efficient at translocation of Ags to the classical MHC I processing pathway (Segura et al., 2009); and finally they express high level of TLR3 which is an enhancer of cross-presentation (Schulz et al., 2005).

## **2.2 T cell fates: priming, peripheral tolerance and exhaustion**

Naïve T cells recirculate continuously between the blood and the SLOs until they encounter their antigen. Signaling through the TCR can have different consequences that range from differentiation into effector and subsequent memory cells, tolerance induction to exhaustion. The strength of the

TCR signal is determined by the number of peptide-MHC complexes and costimulatory molecules on the APC (Lanzavecchia and Sallusto, 2002), whereas cytokines modulate the quality of the ensuing T cell response. Although other cell types including B cells (Gonzalez et al., 2009), macrophages (Backer et al., 2010; Delamarre et al., 2005) and stromal cells, like fibroblastic reticular cells (Fletcher et al., 2010; Lee et al., 2007), can present antigen to T cells, activated or mature DCs are unchallenged in their capacity to activate naïve T cells (Steinman and Nussenzweig, 2002). However, immature DCs are major inducers of peripheral T cell tolerance under steady state conditions and therefore crucial to prevention of autoimmunity (Hawiger et al., 2001; Probst et al., 2003).



**Figure 6. Basic illustration of cross-priming.** Dendritic cells (DCs) present the antigen to CD4<sup>+</sup> T helper (T<sub>H</sub>) cells through MHC class II molecules and cross-present it to CD8<sup>+</sup> cytotoxic T lymphocytes through MHC class I molecules. Mature DCs upregulate expression of co-stimulatory molecules, such as CD70, CD80 and CD86. TLR ligands further activate DCs and increase their cross-presentation activity. T<sub>H</sub> cells can stimulate CTLs through the production of IL-2, (adapted from (Kurts et al., 2010)).

Infection, inflammation and adjuvants result in production of PAMPs or DAMPs that are sensed by DCs via a vast array of PRR and result in functional and phenotypic changes of DCs, a process termed DC maturation (**Figure 6**). TLRs are responsible for DCs polarization and promotion of different type of T cell response, including regulatory T cell response (Kapsenberg, 2003). During the interaction between mature DCs and T cells the immunological synapse is formed, which contains TCR/peptide-MHC complexes and cosignaling molecules (Friedl et al., 2005). Choudhuri and colleagues provided a new model for the immunological synapse: the tumour susceptibility gene 101 (TSG101) sorts TCRs for inclusion in microvesicles that subsequently bud at the center of the immunological synapse. Vacuolar protein sorting 4 (VPS4) mediates scission of microvesicles from the T-cell plasma membrane (Choudhuri et al., 2014), which may explain TCR downregulation upon cognate interactions. Productive activation of naïve T cells requires three signals (Marchingo et al., 2014). Signal 1 is delivered through the TCR and guarantees antigen-

specificity of the response. Without concomitant signals 2 and 3, however, signal 1 promotes T cell unresponsiveness (anergy, peripheral T cell tolerance). Signal 2 consists of co-stimulatory and co-inhibitory signals that are integrated by the T cell to define the ultimate type of response. The prototype co-stimulatory interaction consists of CD28 on the T cell and CD80/CD86 on the APC. CD28 was the first co-stimulatory molecule that was identified and was shown to be crucial to the production of the T cell growth factor IL-2 (Linsley et al., 1991). A variety of stimuli can quickly upregulate CD80 and CD86, otherwise constitutively present on the DCs, which are early costimulatory signals. The complexity of co-stimulation is illustrated by the fact that CD80/CD86 also interact with a co-inhibitory molecule on T cells, like CTLA-4. The latter binds CD80/CD86 with higher affinity than CD28 and it provides a negative feedback loop that down regulates T cell response (Carreno et al., 2000; Greene et al., 1996). ICOS is another co-stimulatory molecule expressed on CD4<sup>+</sup> T cells which depending on the inflammatory environment drives T cell polarization (Kopf et al., 2000). CD8<sup>+</sup> and CD4<sup>+</sup> requires differential co-stimulatory molecules in their responses, indicating distinct mechanisms involved in developing effector (Kaeche et al., 2002). Signal 3 is the polarizing signal delivered from the APCs to the T cells, which usually consists of cytokines and determines in which type of effector cell T cells will differentiate (Chen and Flies, 2013). For example, IL-12 promotes differentiation of T<sub>H</sub>1 cell or cytotoxic T lymphocytes (CTLs) (Trinchieri, 2003) and type I IFN supports clonal expansion and differentiation of naïve CD8 T (Curtsinger et al., 2005).

Naïve CD4 T cells require a minimum of 6 hours (Iezzi et al., 1998) and naïve CD8<sup>+</sup> T cells 2-24 hours (van Stipdonk et al., 2001) of antigenic stimulation to commit to clonal expansion and effector differentiation. The signal through the TCR is driving T cells through a hierarchical threshold of differentiation determining the strength of the response (Lanzavecchia and Sallusto, 2002). Activated, proliferating T cells leave the LN and undergo different fates, depending on the migratory capacities. T cells expressing CCR7 and L-selectin have access to T cell areas of the LN, where they can be further stimulated by the survival-promoting cytokines such as IL-2, IL-7 and IL-15. By contrast, T cells with tissue-homing capacity can migrate to inflamed tissues, where they complete their differentiation (Roman et al., 2002). After activation and proliferation, the majority of T cells die, leaving behind an heterogeneous pool of memory T cells, which can provide life-long protection. The original model was elaborated by Sallusto and colleagues showing that memory T cells circulate in the bloodstream as two distinct subsets: Central memory (T<sub>CM</sub>) T cells that express CCR7, CD62L (L-selectin), can proliferate produce IL-2, and effector memory T cells (T<sub>EM</sub>) that do not express CCR7 or CD62L, are less proliferative and produce IFN $\gamma$  (Sallusto et al., 1999). T<sub>CM</sub> are predominant in the SLOs and T<sub>EM</sub> are predominant in the peripheral compartments (Masopust et al., 2001). Recently, a third subpopulation of memory T cells was identified, the tissue-resident memory T cells (T<sub>RM</sub>) that permanently reside in tissues and mediate local protection (Gebhardt et al., 2009). Differently distributed memory T cells provide a fast and

enhanced protection from reencounter of the same antigens (Schenkel et al., 2013; Sheridan et al., 2013).

T cells chronically exposed to antigens, for example during chronic viral infection or within tumor microenvironments, may adopt an altered phenotype characterized by a reduced proliferative capacity and effector function (Wherry, 2011). These T cells have been termed “exhausted” T cells because they do not respond to ongoing stimuli. This phenomenon was first identified in CD8<sup>+</sup> T cells (Moskophidis et al., 1993), but also CD4<sup>+</sup> T cells can develop an exhausted phenotype (Oxenius et al., 1998). One well-defined mechanism associated with exhaustion is the upregulation of inhibitory molecules like PD-1, CTLA-4 and TIM3 (Fourcade et al., 2012; Wherry, 2011). IL-10 and TGFβ have been associated with exhaustion too (Tinoco et al., 2009). The final stages of exhaustion are the lack of the ability to produce IFN-γ and the elimination of effector cells (Moskophidis et al., 1993; Wherry, 2011). T cell exhaustion, besides interfering with effector function, can prevent the development of highly functional memory T cells (Shin and Wherry, 2007). Interestingly, T cell exhaustion might be rescued blocking inhibitory molecules, cytokines or through costimulatory signals. For example, it has been shown in a model of chronic infection with lymphocytic choriomeningitis virus (LCMV) that an agonist monoclonal antibody specific for 4-1BB (transmembrane glycoprotein belonging to the TNF expressed on activated T cells) in combination with IL-7, can restore the activity of dysfunctional CD8<sup>+</sup> T cells (Wang et al., 2012). Moreover, blockade of PD-1 was described as promising in rescuing T cells function in chronic infection and cancer (Pauken and Wherry, 2015; Sakuishi et al., 2010). These findings are the prove of principle that exhausted T cells are not completely terminal. Reverting the exhausted phenotype of T cells might be a promising approach for enhancing immunity during chronic insults.

There is ample evidence that immature DCs in the steady state tolerize CD4<sup>+</sup> and CD8<sup>+</sup> T cells by inducing deletion or tolerance (Steinman et al., 2003; Steinman and Nussenzweig, 2002). This represent an homeostatic system to take under control the peripheral T cell repertoire of autoreactive T cells that escaped thymic deletion, contributing in limiting autoimmunity (Steinman and Nussenzweig, 2002). DCs which have not sensed PAMPs and/or DAMPs through TLRs or other sensor molecules, do not mature completely. Consequently, instead of breaking the peripheral tolerance of T cells, they present MHC/peptide complexes that cause T cells anergy (process termed cross-tolerance), because of the lack of costimulatory molecules (Kurts et al., 1997; Probst et al., 2005; Steinman et al., 2003). DCs tolerogenicity is not specific to a single subsets and it can also be induced and maintained by various anti-inflammatory and immunosuppressive agents, including IL-10 (Morelli and Thomson, 2007). Additionally, co-inhibitory molecules can provide negative signals that inhibit T cells response, mediate T cells tolerance, preventing autoimmunity. These receptors are programmed cell death 1 (PD-1) and cytotoxic T lymphocyte (CTL)-associated antigen 4 (CTLA-4) and their ligands are respectively PD-L1/2 and CD80/CD86. It has been

demonstrated that resting, antigen-presenting DCs induce tolerance through the engagement of PD-1 and CTLA-4 (Probst et al., 2003; Probst et al., 2005). In the absence of the signal 2, signal 1 from APCs induces in T cells a state of long-term hyporesponsiveness termed “anergy”. T<sub>regs</sub> have been postulated to promote an hypoxic environment able to regulate T cells by anergy (Sitkovsky, 2009). Moreover, lymph nodes stromal cells, like fibroblastic reticular cells (FRCs), follicular DCs and lymphatic endothelial cells (LECs), can also present antigens and induce tolerance (Cohen et al., 2010).

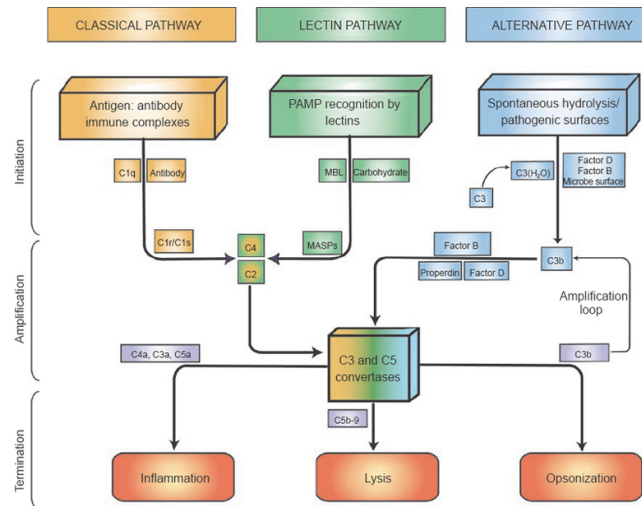
### **2.3 The complement system: a bridge between innate and adaptive immune response**

Complement is an ancient defense mechanism and is considered a bridge between innate and adaptive immunity (Zhu et al., 2005). The complement system consists of more than 30 soluble and surface proteins and can be activated through 3 different pathways: the classical, lectin and alternative pathway. The classical pathway starts when C1q binds to an immune complex (antigen-antibody), subsequently activates C1s and C1r and ultimately results in the cleavage of C4 and C2. The lectin pathway is initiated when mannose-binding lectin (MBL) binds carbohydrate motifs that are found on bacteria and subsequently activates the MBL-associated serine proteases (MASPs), leading to cleavage again of C4 and C2. In both the lectin and classical pathways the cleavage products of C4 and C2 form C3 convertase, which cleaves the central protein of the complement system (C3) into C3b, C3c and C3a. C3b associates with C4bC2a complex to form C5 convertase of the classical and lectin pathway. The alternative pathway is activated when C3 undergoes spontaneous hydrolysis in the presence of Factor B and D and the subsequent formation of the C5 convertase of the alternative cascade (C3bBbC3b). The final step is the formation of the membrane attack complex (MAC) (**Figure 6**), which is involved in lysis of target cells.

The anaphylatoxins C3a and C5a are potent pro-inflammatory mediators that act as opsonins by binding the surface of target cells that need to be eliminated by phagocytes (Walport, 2001a; Walport, 2001b). Because of its destructive potential, complement activation is tightly regulated by soluble (factor H) and membrane-bound inhibitors (CD46, CD55 and CD59) that interfere with the activation of convertases or the formation of the MAC (Liszewski et al., 1996).

Complement receptors are expressed by many cell types, including leukocytes. It has been demonstrated that upon infection the priming of CD4<sup>+</sup> and CD8<sup>+</sup> T cells is reduced in the absence of C3 (Kopf et al., 2002). Moreover, anaphylatoxins are crucial to T cell costimulation because they enhance costimulatory signals on DCs (Strainic et al., 2013). In fact, C5a receptor signal transduction in T cell is important to allow optimal T-cell expansion, as well as to maintain viability (Lalli et al., 2008). Recent studies demonstrated that anaphylatoxins are crucial mediators during the cognate interaction between DCs and naïve CD4<sup>+</sup> T cells without which T cells develop into Tregs instead of effector cells (Le Friec et al., 2013; Strainic et al., 2013). Anaphylatoxins

(C3a and C5a) are products of the cleavage of complement factor 3 (C3) and complement factor 5 (C5) and can be generated by both DCs and T cells (Strainic et al., 2013).



**Figure 6. The 3 pathways of the complement system.** Complement can be activated through the classical, alternative and lectin pathway. The initiation phase is mediated but different factors specific for each pathway. The 3 pathways culminate with the production of C3 and C5 convertases that leads to the production of factors involved in inflammation (anaphylatoxins: C3a, C5a, C4a); lysis of target cells (MAC) and opsonization (C3b, C3bi, C4b), (Dunkelberger and Song, 2010).

The complement system contains some of the most powerful proinflammatory molecules. Acute inflammation is a host response to infection, injury or disturbance that lasts until the insult is cleared. When acute inflammation becomes dysregulated, excessive or chronic it can lead to several pathologies. There is a variety of inflammatory disorders like arthritis, autoimmune diseases, asthma, ischemia/reperfusion injuries and cancer.

Complement plays an important and mostly beneficial role in acute inflammation as it contributes to resolution of the disturbance (Guo and Ward, 2005; Markiewski and Lambris, 2007). In contrast, complement aggravates the detrimental consequences of chronic inflammation and the use of complement inhibitors in chronic inflammatory diseases is currently being tested in preclinical models and clinical trials (Ricklin and Lambris, 2013).

Cancer is linked with chronic inflammation but a role of complement in cancer initiation or progression is controversial. On the one hand, there is evidence for the presence of activated complement in cancer patients (McConnell et al., 1978) and for a tumor-promoting role of complement via immune suppression (Markiewski et al., 2008). On the other hand, tumor cells express complement regulators in order to escape the control mediated by complement (Li et al., 2007) and production of anaphylatoxins at low levels has been related with reduced tumor growth and better immune response (Gunn et al., 2012). One possible hypothesis is that complement,



activated in a chronic fashion, might create a selective pressure in the tumor microenvironment causing tumor growth (Pio et al., 2013); instead acute activation of the complement pathway may lead to improved immune response and tumor control (Surace et al., 2015).

### **3. Tumor Immunology**

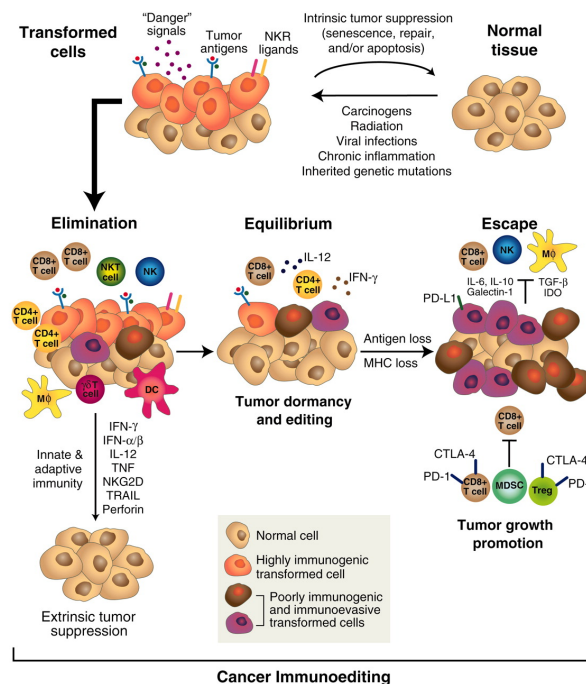
#### **3.1 Immunity against cancer**

It is accepted now that the immune system detects and can control cancer. Paul Ehrlich was the first in the early 1900s to propose involvement of the immune system in tumor control. Based on observations in animal models, Burnet and Thomas as well as Old proposed the theory of “immune surveillance of cancer” in the 1950s, speculating that lymphocytes detect tumor antigens on transformed cells to destroy them (Burnet, 1957; Old and Boyse, 1964; Thomas, 1982). However, Stutman’s observations in 1975 that athymic nude mice did not have increased susceptibility to chemically induced tumors almost destroyed the field of tumor immunology (Stutman, 1974). Today we know that nude mice have residual numbers of T cells as well as high numbers of NK and other immune cells (Dunn et al., 2004). This notion together with the discovery of tumor necrosis factor (Carswell et al., 1975) and the identification of the genes encoding tumor antigens recognized by T cells (van der Bruggen et al., 1991), revived the tumor immunology and the involvement of the immune system in cancer is now a well established concept (Gajewski et al., 2013). The basic idea of tumor immunology is that cancer cells express antigens that discriminate them from their non-transformed counterparts (Cheever et al., 2009; Old, 1981). Besides protecting the host against cancer, it is quite clear that the immune system also shapes the immunogenicity of the tumor in a dynamic process called cancer immunoediting (Dunn et al., 2004; DuPage et al., 2012; Schreiber et al., 2011; Vesely et al., 2011). Cancer immunoediting involves 3 phases termed elimination, equilibrium and escape and is therefore often referred to as the 3E-concept (**Figure 7**). The elimination phase depends on danger signals such as type I IFNs, damage-associated molecular patterns (DAMPs), stress ligand (like RAE-1 and H60) for the stimulation of innate and adaptive immune cells (Sims GP, Ann Rev Imm 2010). Studies in knock out mice showed that IFN- $\gamma$ , perforin, Fas/FasL, TRAIL, and NKG2D are fundamental in the elimination phase. Recent studies showed that type I IFNs are required for initiation in the initial phases because they enhance cross-presentation by CD8 $\alpha^+$ CD103 $^+$  DCs (Fuertes et al., 2011). One problem with studying the elimination phase is to distinguish between tumors that were eliminated and tumors that were never there. Good models for elimination are still missing, but all evidence points towards a crucial role for CD8 $^+$  T cells and NK cells (Biroccio et al., 2013; Gasser et al., 2005).

If tumor cells survive the elimination phase, the equilibrium phase may occur. The immune system controls the outgrowth of the tumor, maintaining tumor cells in a state of dormancy and in parallel shaping the immunogenicity. Existence of the equilibrium phase has been assumed for considerable time but was difficult to prove until a seminal study showed that interfering with adaptive immunity resulted in outgrowth of stable masses of transformed cells in mice (Koebel et al., 2007). It should be noted that it is unclear which proportion of cancers are fully eliminated or kept in equilibrium by the immune system, because such events are clinically unnoticed.

In the escape phase, tumor cells have acquired the ability to circumvent the immune control by different mechanisms including selection of tumor cells that lack expression of rejection antigens of MHC, inefficient antigen presentation to tumor-specific T cells and the promotion of an immunosuppressive environment (Schreiber et al., 2011).

CD8<sup>+</sup> T cells are key players in each single phase of the cancer immunoediting process. High numbers of CD8<sup>+</sup> tumor infiltrating lymphocytes (TIL) correlates with increased survival in patients with solid tumors (Fridman et al., 2012). Tumors arising in *Rag2*<sup>-/-</sup> mice (lacking T and B cells) are more immunogenic than those derived from an immunocompetent host, which explained by selective outgrowth of escaped tumors in immunocompetent mice (DuPage et al., 2012; Schreiber et al., 2011). Experiments comparing *Rag2*<sup>-/-</sup> and *Rag2*<sup>-/-</sup> x *γc*<sup>-/-</sup> (lacking also NK cells), demonstrated that innate cells present in *Rag2*<sup>-/-</sup>, but not in *Rag2*<sup>-/-</sup> x *γc*<sup>-/-</sup> mice, also may contribute to shaping immunogenicity of a tumor (O'Sullivan et al., 2012).



**Figure 7. The cancer immunoediting concept.** After cellular transformation has occurred and intrinsic tumor suppression mechanism have failed to control tumor development; an additional extrinsic mechanism takes place and this is called immunoediting. It consists of 3 phases: elimination, equilibrium and escape.

Transformed cells can be recognized by the immune system and destroyed in a phase called elimination. If a rare cell variant survive this process, it may enter an equilibrium phase in which different immune cell types and molecules collaborate to maintain the tumor in a state of dormancy. In this phase the editing process happens. However, due to the constant immune selective pressure, tumor cell variants that are no longer recognized or that create a suppressive environment can emerge leading to the outgrowth of the tumor. These tumor cells enter the escape phase in which their development is no longer under the control of the immune system. At this stage the tumor became clinically apparent (Schreiber et al., 2011).

### **3.2 The major stumble stones for immunity against cancer**

The major problems for immune control of cancer are central tolerance to tumor-associated antigens resulting in absence of high-affinity tumor-specific T cells, insufficient innate stimulation resulting in defective T cell activation and cancer-associated immunosuppression resulting in blunted protective immunity (Baitsch et al., 2012).

The recognition of tumor-associated antigen (TAA) presented by MHC on tumor cells or APCs is a fundamental step for T cell-mediated tumor control. TAA can be divided in neoantigens, which originate from viruses or mutations, and self-antigens that are overexpressed host proteins (Coulie et al., 2014). It was thought for a long time that central tolerance was limited to ubiquitously expressed proteins, however, Kyewski and colleagues showed that all proteins including tissue-specific proteins are expressed by medullary thymic epithelial cells and induce central tolerance (Kyewski and Klein, 2006; Kyewski et al., 1984). Similar data were shown for human TAA (Gotter et al., 2004). This has consequences for the quality of most tumor-specific T cells because the peripheral T cell repertoire will be purged of self-reactive T cells with a high-affinity TCR (Redmond and Sherman, 2005).

Although spontaneous tumor-specific responses are observed in some cancer patients, the effector function of such T cells often is compromised. Insufficient innate stimuli lead to defective DC maturation thus precluding proper T cell priming. Especially tumor-associated DCs seem tolerogenic as they express low amounts of MHC and co-stimulatory molecules and produce the immunosuppressive cytokine IL-10 (Gabrilovich, 2004). Growing tumors progressively contain more immunosuppressive factors and less pro-inflammatory signals that impair DC maturation and development or maintenance of protective immunity (Bell et al., 1999; Troy et al., 1998; Zou et al., 2001), which also seems to be the case for tumor-draining lymph nodes (Fujita et al., 2009). Many tumors upregulate vascular endothelial factor C (VEGF-C), which increases lymphangiogenesis causing an increased delivery of TAAs (Pepper, 2001). The prolonged exposure to TAAs in the absence of sufficient innate stimuli may cause exhaustion of T cells (Bucks et al., 2009; Hansen et al., 2007). Moreover, lymphatic endothelial cells (LECs) can take up and present TAAs to CD8<sup>+</sup> T

cells. Since LECs lack costimulatory molecules and express PD-L1, this causes tolerization of T cells (Lund et al., 2012).

Even if tumor-specific effector T cells are produced and manage to infiltrate the tumor, the immunosuppressive microenvironment seriously hampers protective immunity and tumors have several mechanisms at their disposal to evade immune attack. Tumor cells escape recognition of CTLs by downregulating MHC class I molecules (Fonsatti et al., 2003; Meissner et al., 2005) and by expressing different or less immunogenic antigens (Khong and Restifo, 2002). Besides avoiding the recognition by T cells, the tumor microenvironment impairs immune infiltration by altering the expression of adhesion molecules such as E-Selectin and P-Selectin (Weishaupt et al., 2007). High levels of TGF- $\beta$  have been associated with unfavorable prognosis in different malignancies (Curiel et al., 2004; Drake et al., 2006). TGF- $\beta$  controls T cell homeostasis by inhibiting activation, proliferation and differentiation (Kehrl et al., 1986). IL-10, together with other factors, blocks DC maturation, lymphocyte function and promotes the recruitment of Tregs and myeloid-derived suppressor cells (MDSCs) (Bergmann et al., 2007; Serafini et al., 2006). T<sub>regs</sub> and MDSCs impair T cell function through direct contact or by secretion of immunomodulatory molecules. MDSCs produce reactive oxygen species (ROS), which impair the TCR interaction with the MHC/peptide complex and induce T cell tolerance locally in the tumor (Nagaraj et al., 2007). High numbers of T<sub>regs</sub> in the tumor correlates with reduced survival in most human cancers analyzed (Curiel et al., 2004; Kono et al., 2006) and selective depletion of Tregs results in CD8<sup>+</sup> T cell-mediated tumor rejection (Carretero et al., 2015; Turk et al., 2004). Moreover, inhibitory coreceptors, such as CTLA-4 and PD-1 are crucial to peripheral T cell unresponsiveness. Mice deficient for CTLA-4 or PD-1 spontaneously develop autoimmunity (Nishimura et al., 1999; Tivol et al., 1996; Waterhouse et al., 1995) suggesting that these molecules are crucial to maintenance of self-tolerance. Allison and colleagues were the first to demonstrate that blockade of CTLA-4 enhances antitumor immunity (Leach et al., 1996), although the underlying mechanism was not identified at that time. Blockade of PD-1 restored the effector function of exhausted T cells in murine and human chronic viral infection with lymphocytic choriomeningitis virus (LCMV) (Barber et al., 2006) and human immunodeficiency virus (HIV) (Day et al., 2006; Freeman et al., 2006), respectively. Tumor cells upregulate PD-L1 on their surface as an immune escape mechanism (Dong et al., 2002; Hatanishi et al., 2007). Hypoxia, a general condition in many tumors, supports the recruitment of Tregs while stimulating the production of adenosine, which lowers CD8<sup>+</sup> T cells activity (Facciabene et al., 2011).

### **3.3 Exploiting the immune system to control cancer**

With hindsight, the first immunotherapy was performed by William Coley in 1890s, a New York surgeon, who mainly operated patients with sarcoma. He observed remission in a cancer patient

upon development of erysipelas, a severe skin infection with *Streptococcus pyogenes*. Subsequently, Coley injected cancer patients with heat-killed *S. pyogenes* plus *Serratia marcescens*, a mixture called Coley's toxin, and observed remission in some cases (Coley, 1891). Helen Coley Nauts, Coley's daughter, later tabulated around 1000 patients treated by the father. She noticed that 500 of these cases showed near-complete remission (Nauts and McLaren, 1990). Despite the success of Coley's toxin, it met criticism at that time and it suffered the pressure from the developing fields of radiotherapy and chemotherapy. Nevertheless, Coley's studies inspired the use of bacille Calmette-Guerin (BCG) as immunotherapy, still in use as the most effective treatment against superficial bladder cancer (Dranoff, 2004; Herr et al., 1995).

To date, different immunotherapeutical approaches are in clinical trials and some have been recently approved in multiple countries. These include passive therapies such as antibodies or T cells targeting tumor cells as well as active therapies that aim to stimulate the patient's immune system such as immunization, DC activation and blocking co-inhibition (checkpoint blockade).

The problem of insufficient T cell priming or a suboptimal repertoire in the context of cancer can be circumvented by adoptive transfer of properly activated, high-affinity T cells. These include the adoptive transfer of *in vitro* activated and expanded, autologous tumor-infiltrating lymphocytes (TILs) together with IL-2 (Dudley et al., 2002), T cells activated with defined melanoma tumor antigens (Hunder et al., 2008), T cell transduced with high-affinity TCRs that specifically recognize TAAs (Morgan et al., 2006) and T cells transduced with chimeric antigen receptors (CARs) recognizing tumor-specific cell surface proteins that are composed of hybrid immunoglobulin light chains with endo-domains of T cells signaling molecules (Kalos et al., 2011). These approaches have shown remarkable clinical responses especially using CD19- or CD20-specific T cells to treat lymphomas.

Cancer vaccines intend to treat existing malignancies or prevent their formation. The vaccine against human papilloma virus (HPV) is the most successful cancer vaccine to date that works prophylactically but also showed efficacy when used as a therapeutic vaccine (Kenter et al., 2009) in patients with cervical cancer and vaginal neoplasias. The fact that the vaccine is based in a viral antigen may explain the efficacy. Another, less successful, example is Provenge (a treatment approved by the FDA in 2010 for metastatic prostate cancer, sipuleucel-T), which is a preparation of patient's peripheral blood-derived APCs, loaded with a fusion protein of granulocytes-macrophage colony stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP). Upon injection the GM-CSF-activated APCs in the preparation should present PAP to patient's T cells inducing their activation and proliferation (Kantoff et al., 2010). Treatment with a vaccine consisting of modified gp100 peptide (a melanocyte-specific protein) plus IL-2 resulted in increased survival of melanoma patients when compared to IL-2 alone (Schwartzentruber et al., 2011). The principle for the above-mentioned vaccines is the combination of a known tumor

antigen and a factor to stimulate APCs or T cells. Unfortunately, characterized antigens are not available for many tumors, so this approach is not always applicable.

Mature DCs are the most potent cells for stimulation of memory and naive T cells. The administration of ligands for TLRs have shown potent anticancer effect against established tumors in both mice and humans (Krieg, 2007). For example, local application of the TLR7 agonist Imiquimod to superficial basal cell carcinoma resulted in cure in 80-90% of cases (Schulze et al., 2005). However, systemic administration of Imiquimod in patients with more advanced cancers has not resulted in the same degree of success (Dummer et al., 2008), nor was it proven that DC activation indeed was crucial to the therapeutic efficacy. Despite their limited clinical success as monotherapies, the ability of TLR agonists to activate DCs makes them good candidates for combination with peptide/protein vaccines (Speiser et al., 2005) or with chemotherapy that presumably increases the presentation of tumor antigens (Guha, 2012). DC maturation can efficiently be achieved by agonistic anti-CD40 antibodies (Sarawar et al., 2001; Schoenberger et al., 1998) and such antibodies showed potent anti-tumor effects in several mouse models. However, anti-CD40 used as monotherapy showed only modest clinical activity in cancer patients (Advani et al., 2009). In addition, anti-CD40 showed considerable side effects in patients and therefore, the development of therapies based on anti-CD40 lags behind that of other immunotherapies.

Nowadays, the development of a new class of immunotherapies has become possible because of a better understanding of T cell function and activation. T cell activation is tightly regulated by inhibitory receptors such as CTLA-4 and PD1 in order to avoid inadequately strong immune responses (Fourcade et al., 2012; Wherry, 2011). The first antibody that targets one of the inhibitory receptors on T cells, CTLA-4, was approved by the FDA for the treatment of metastasized melanoma in 2010 (Ipilimumab or Yervoy) and caused an explosion of the field. It is not exactly understood how anti-CTLA-4 works, but recent preclinical data suggest selective depletion of tumor-associated Tregs as a mechanism (Simpson et al., 2013). Tumors frequently express the ligands for PD-1, PD-L1 and PD-L2 and this expression can be induced by IFN- $\gamma$  (Twyman-Saint Victor et al., 2015). PD1 ligation interferes with TCR signaling (Greenwald et al., 2005). Blockade of PD1/PDL1 axis showed impressive results in the clinics for the treatment of different tumor types (Powles et al., 2014; Topalian et al., 2012). Since PD1 and CTLA-4 are interfering with different pathways, in 2013 a clinical trial with the combination of anti-CTLA4 and anti-PD1 started in melanoma patients that showed tumor regression in 50% of the patients (Wolchok et al., 2013). Also the combination of checkpoint blockade with cytokines seems promising. For example, the combination of anti-CTLA-4 with IL-12 induced regression of established gliomas in mice (Vom Berg et al., 2013) and a clinical trial is now being planned. Injection of IL-2/anti-IL-2 immune complexes selectively stimulates effector T cells without promoting Treg proliferation (Krieg et al., 2010) and resulted in control of B16 melanomas. This

effect was further improved if anti-CTLA-4 was added to the therapy. A clinical trial with melanoma patients using this combination is planned.

Despite the clinical efficacy of checkpoint blockade, only a proportion of patients respond. Recent data show that especially patients with highly mutated tumors respond well, suggesting that mutations result in the expression of novel epitopes that are more immunogenic (Gubin et al., 2014; Kreiter, 2014). Furthermore, de novo priming rather than boosting pre-existing immunity seems a major consequence of immunotherapy (Bransi et al., 2015; Rizvi et al., 2015; Schumacher and Schreiber, 2015).

Taken together, the field of cancer immunotherapy is rapidly evolving because of major achievements in recent years. Many trials are currently ongoing in which different immunotherapies are combined with each other or with standard therapies such as chemo- or radiotherapy.

## **4. Radiotherapy**

### **4.1 Radiotherapy induces lethal DNA damage**

In the late 19<sup>th</sup> century the discovery of ionizing radiation was awarded with three Nobel prizes. In 1948 the first radiation machine (cobalt source) was built and the first cancer patients were treated 6 months after (Grubbe, 1946). The clinical efficacy of radiotherapy became quickly apparent and research concentrated on achieving maximal efficacy with minimal side effects since then (Thariat et al., 2013). Technological advancements allowed moving from the first megavoltage linear accelerator X-rays machine to the computer-modulated external beam radiotherapy (ERBT), the stereotactic body radiotherapy (SBRT), and finally high-dose radiotherapy and proton therapy (Allen et al., 2012). Nowadays, the field is moving towards hypofractionated radiotherapy, meaning application of radiation at high doses in fewer fractions (Vaidya et al., 2010).

The efficacy of radiotherapy was thought to rely on its capacity to damage nucleic acids, protein and lipids, which is amplified by the radiation-mediated production of free radicals. Double-strand breaks (DSBs) and single-strand break (SSBs) are the most frequent types of damage to which cancer cells are more susceptible than normal tissue because of frequent mutations in DNA repair pathways (Begg et al., 2011; Giusti et al., 1998; Negrini et al., 2010). Cells exposed to ionizing radiation can undergo apoptosis, necrosis, mitotic catastrophe, autophagy and senescence. The type of cell death strictly depends on the cell type, the dose delivered, the cell cycle phase, the redox state and the DNA repair capacity of the target cell (Eriksson and Stigbrand, 2010; Golden et al., 2012). Although the type of cell death matters to the immune system (Kroemer et al., 2013), this issue is not well investigated yet.

## 4.2 Radiotherapy stimulates tumor-specific immunity

Recent evidence demonstrated that the direct killing of tumor cells is not the only mode of action of radiotherapy. It is now clear that radiotherapy-induced immunogenic cell death (ICD) and the stimulation of tumor-specific immunity are integral parts of its therapeutic efficacy (Apetoh et al., 2007; Formenti and Demaria, 2012; Gupta et al., 2012; Sharma et al., 2013).

Numerous studies highlighted different mechanism by which radiotherapy can overcome some of the major stumble stones described above.

Studies showed that radiotherapy increases the amount and variety of tumor-associated antigens expressed by tumor cells, permitting APCs like DCs to prime T cells for specific recognition and killing of tumor cells (Sharma et al., 2013). Furthermore, radiation increases the expression of MHC class I molecules by tumor cells *in vivo* and *in vitro*, which makes them visible to CD8<sup>+</sup> T cells (Reits et al., 2006; Sharma et al., 2013). Moreover, DCs recognize calreticulin exposed on the surface of tumor cells upon radiation as an eat-me signal (Obeid et al., 2007). It has been reported that radiotherapy leads to the release of high-mobility group box 1 (HMGB1) by irradiated tumor cells. HMGB1 is a potent DAMP that sustains DCs activation through activation of TLR-4 leading to an efficient T cell response (Apetoh et al., 2007). Radiation induces DCs maturation (Gupta et al., 2012) and increases the trafficking of DCs towards the draining lymph nodes, where they present the antigen to the T cells (Lugade et al., 2005). The infiltration of CD8<sup>+</sup> T cells in the tumor is also enhanced by radiotherapy and it stimulates the production of type I and II IFNs, thus enhancing cross-presentation (Fuentes et al., 2011; Gupta et al., 2012; Lim et al., 2014). The tumor microenvironment also undergoes changes upon radiotherapy. Tumor-associated blood vessels are normalized upon radiotherapy, facilitating the infiltration of immune effectors (Huang et al., 2013). Radiotherapy causes the loss of lymphatic vessels and it impaires their function (Avraham et al., 2010).

Nevertheless, radiotherapy has also immunosuppressive effects. Radiation causes the production of ROS that amplify the inflammatory cascade (Valerie et al., 2007) and may result in tumor-promoting chronic inflammation. T<sub>regs</sub> increase upon radiotherapy and contribute to tumor development and radioresistance (Formenti and Demaria, 2013). In addition, it has been reported that radiotherapy leads to an increased production of co-inhibitory molecules like PD-L1 on tumor cells upon RT (Deng et al., 2014).

The net impact of radiotherapy on tumor-specific immunity may depend on the therapeutic protocol, but data are limited and conflicting. For example, it has been suggested that high-dose radiotherapy is more potent in inducing immune-responses than low-dose radiotherapy (Lee et al., 2009). Further studies are needed to clarify the effects of different radiotherapy protocols. In contrast, lower doses seemed to better synergize with anti-CTLA-4 (Dewan et al., 2009).



Taken together, radiotherapy is well established in the clinics and responsible for about 40% of cancers that are cured. Despite this, fundamental knowledge of pathways affected by radiotherapy and precise understanding of the effects on tumor-specific immunity are scarce. Also comparisons between different radiotherapy protocols, especially between hypo- and hyperfractionated radiotherapy, in which clinical and biological parameters are linked are hardly performed.

We reasoned that some radiotherapy-induced pathways will support tumor-specific immunity and thus clinical efficacy, whereas others will suppress. Specific interference with such pathways may increase the efficacy of radiotherapy and will provide a rational basis for novel combination therapies.

Therefore, my work has following aims:

1. Investigation of the upstream mechanism leading to the radiotherapy-mediated tumor-specific immune response
2. Characterization of radiotherapy-induced changes in the tumor-associated lymphatic vessels

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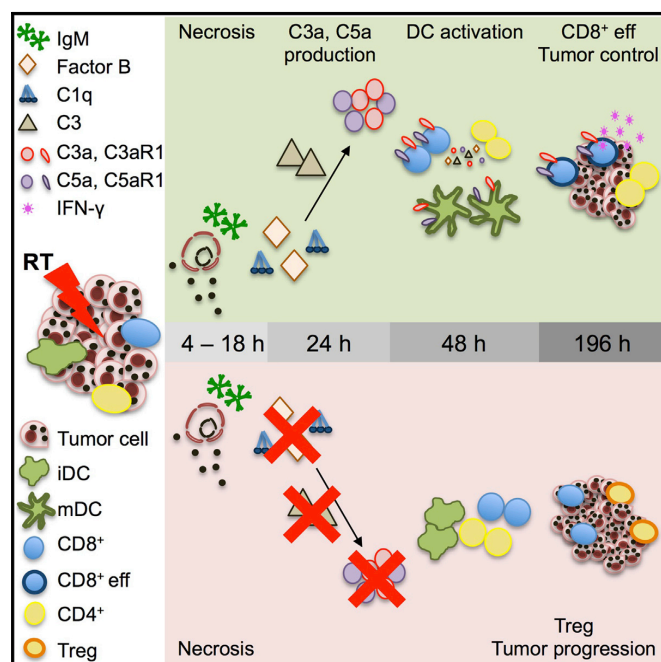


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# Immunity

## Complement Is a Central Mediator of Radiotherapy-Induced Tumor-Specific Immunity and Clinical Response

### Graphical Abstract



### Authors

Laura Surace, Veronika Lysenko, ..., Anurag Gupta, Maries van den Broek

### Correspondence

vandenbroek@immunology.uzh.ch

### In Brief

Anaphylatoxins are produced upon complement activation and are well-known pro-inflammatory molecules. van den Broek and colleagues demonstrate that anaphylatoxins are produced within a tumor after radiotherapy by immune cells, support tumor-specific immunity, and are essential to therapeutic efficacy.

### Highlights

- RT induces local complement activation in mice and humans
- RT-mediated cell death and necrosis activates complement
- C3a and C5a accumulate in the tumor and promote tumor-specific immunity
- Dexamethasone inhibits complement activation and reduces efficacy of RT

Surace et al., 2015, Immunity 42, 1–11  
 April 21, 2015 ©2015 Elsevier Inc.  
<http://dx.doi.org/10.1016/j.immuni.2015.03.009>

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# Complement Is a Central Mediator of Radiotherapy-Induced Tumor-Specific Immunity and Clinical Response

Laura Surace,<sup>1</sup> Veronika Lysenko,<sup>1</sup> Andrea Orlando Fontana,<sup>2</sup> Virginia Cecconi,<sup>1</sup> Hans Janssen,<sup>3</sup> Antonela Bicvic,<sup>1</sup> Michal Okoniewski,<sup>4</sup> Martin Pruschy,<sup>2</sup> Reinhard Dummer,<sup>5</sup> Jacques Neefjes,<sup>3</sup> Alexander Knuth,<sup>6,7</sup> Anurag Gupta,<sup>1</sup> and Mares van den Broek<sup>1,\*</sup>

<sup>1</sup>Institute of Experimental Immunology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

<sup>2</sup>Department of Radio-Oncology, University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland

<sup>3</sup>Division Cell Biology II, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands

<sup>4</sup>ID Scientific IT Services, Swiss Federal Institute for Technology (ETH), Weinbergstrasse 11, 8092 Zurich, Switzerland

<sup>5</sup>Department of Dermatology, University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland

<sup>6</sup>Clinic of Oncology, University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland

<sup>7</sup>Present address: National Center for Cancer Care & Research NCCCR, Hamad Medical Corporation, P.O. Box 3050 Doha, Qatar

\*Correspondence: [vandenbroek@immunology.uzh.ch](mailto:vandenbroek@immunology.uzh.ch)

<http://dx.doi.org/10.1016/j.immuni.2015.03.009>

## SUMMARY

Radiotherapy induces DNA damage and cell death, but recent data suggest that concomitant immune stimulation is an integral part of the therapeutic action of ionizing radiation. It is poorly understood how radiotherapy supports tumor-specific immunity. Here we report that radiotherapy induced tumor cell death and transiently activated complement both in murine and human tumors. The local production of pro-inflammatory anaphylatoxins C3a and C5a was crucial to the tumor response to radiotherapy and concomitant stimulation of tumor-specific immunity. Dexamethasone, a drug frequently given during radiotherapy, limited complement activation and the anti-tumor effects of the immune system. Overall, our findings indicate that anaphylatoxins are key players in radiotherapy-induced tumor-specific immunity and the ensuing clinical responses.

## INTRODUCTION

It is now well accepted that the immune system can control tumors (Schreiber et al., 2011). For example, there is a positive correlation between tumor infiltration by effector T cells and survival (Fridman et al., 2011), and the risk to develop cancer is increased in immunosuppressed patients (Dunn et al., 2006), and dormant tumors are kept in check by the adaptive immune system (Koebel et al., 2007). Despite the presence of tumor-specific immunity in many cancer patients, complete rejection of clinically apparent tumors by the immune system is rare, presumably due to mechanisms that locally inhibit tumor-specific protective immunity (Schreiber et al., 2011). The clinical efficacy of so-called checkpoint blockade, antibodies that target co-inhibitory molecules such as CTLA-4 or PD-1, underscores the potential of tumor-specific immunity (McDermott and Atkins, 2013; Mellman et al., 2011).

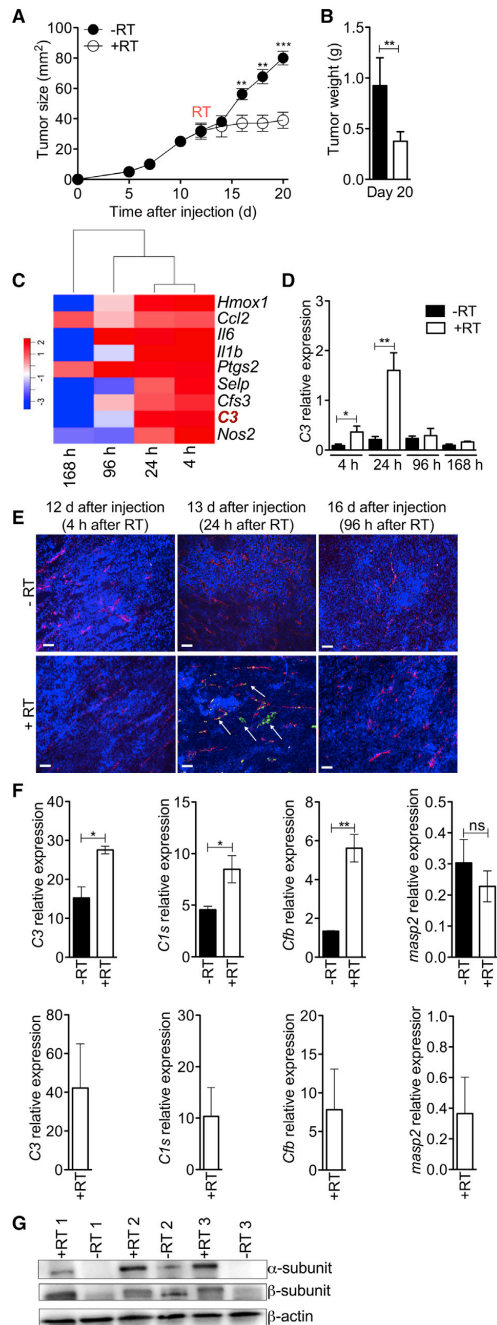
Radiotherapy is a standard treatment for cancer that induces irreversible damage to DNA, thus targeting mainly rapidly dividing cells (Prise and O'Sullivan, 2009). Although radiotherapy was considered an immunosuppressive treatment (Merrick et al., 2005), there is accumulating evidence that it supports local tumor-specific immunity (Apetoh et al., 2007; Matsumura et al., 2008) and, in fact, that immune activation might be an integral part of radiotherapy (Formenti and Demaria, 2012; Gupta et al., 2012; Ma et al., 2010; Sharma et al., 2013). This is clinically relevant because tumor-specific immunity can target dormant lesions (Postow et al., 2012) that are presumably insensitive to radiotherapy. Several studies have addressed the question of how radiotherapy supports tumor-specific immunity, and various factors were suggested, including increased presence or function of tumor-infiltrating CD8<sup>+</sup> T cells (Gupta et al., 2012; Lugade et al., 2005; Takeshima et al., 2010), type I interferon (IFN) resulting in enhanced antigen cross-presentation (Burnette et al., 2011; Fuertes et al., 2011), increased expression of major histocompatibility complex (MHC) class I glycoproteins and tumor-associated antigens (Reits et al., 2006), and maturation of tumor-associated dendritic cells (DCs) (Gupta et al., 2012), however, an initial event has not been identified.

We therefore performed an unbiased analysis of immune response-related transcripts after radiotherapy in a preclinical model and noted a strong and transient upregulation of genes associated with the complement pathway. This was unexpected as complement was described as tumor-promoting (Markiewski et al., 2008; Pio et al., 2014), although other studies have shown that complement supports adaptive immunity (Farrar and Sacks, 2014; Kopf et al., 2002; Lalli et al., 2007; Liszewski et al., 2013; Strainic et al., 2013). Because we observed similar changes in human tumor samples, we investigated the impact of complement on the anti-tumor immune response following radiotherapy.

## RESULTS

### Radiotherapy Induces Complement Activation

To identify the initial event in radiotherapy-induced tumor-specific immunity, we performed an unbiased analysis of immune



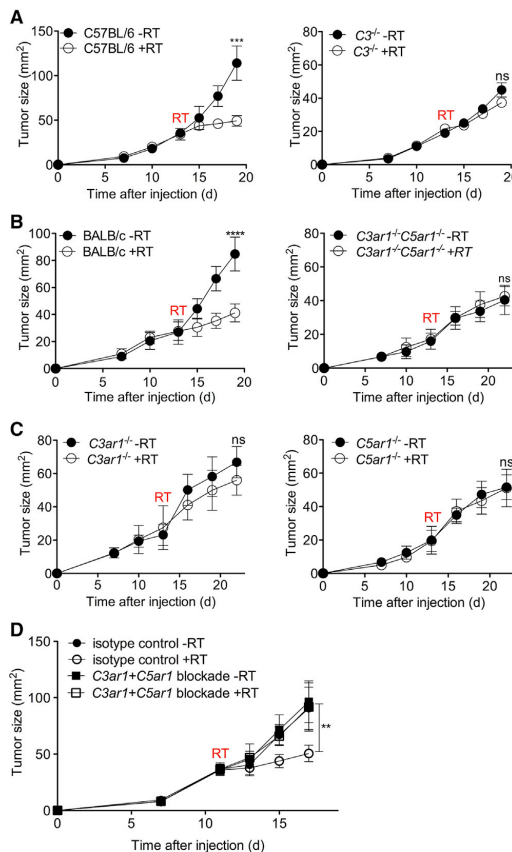
### Figure 1. Radiotherapy Results in Transient Upregulation and Activation of Complement in Murine and Human Tumors

(A–E) Mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. (A) Tumor growth curves, (n = 5 mice per group). Representative data from three independent experiments are shown. (B) Tumor weight (day 20). (C) Heatmaps of transcripts were created using the log<sub>2</sub> value of the fold increase of irradiated compared to untreated tumors at different time points after radiotherapy, (n = 6 mice per time point). Representative data from two independent experiments are shown. (D) Relative expression of C3 mRNA in tumors at different time points after radiotherapy. The data show the mean ± SD of triplicates from three independent analyses. Radiotherapy, RT. (E) Immunofluorescence of irradiated and untreated tumors. Sections were stained with an antibody recognizing C3b, iC3b, and C3c (green), CD31 for blood vessels (red), and DAPI (blue). Scale bars represent 100 μm (n = 6 mice per group). Representative images from two independent experiments are shown.

(F and G) Patient biopsies were collected before and 24–36 hr after radiotherapy (1.5–2 Gy; upper panel) or only after radiotherapy (lower panel). Quantification of complement transcripts by qPCR. Data show the mean ± SD of triplicates from two independent analyses (upper panel). (G) Quantification of C3-α and -β subunits by immuno-blot. Data are shown as mean ± SD. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.0005 by Student's t test (A, B, and F) or two-way ANOVA with the Bonferroni correction (D). See also Figure S1.

response-related transcripts after radiotherapy. Local irradiation with a single dose of 20 Gy significantly reduced progression of B16F10-OVA tumors in C57BL/6 mice (Figures 1A and 1B). Because transplantable mouse tumors only allow for a short therapeutic window, radiotherapy must be applied as a single high dose (Lugade et al., 2005). To dissect which pathways are crucial to radiotherapy-induced stimulation of the immune response, we quantified immune response-related transcripts in tumors at different time points 4, 24, 96, and 168 hr after local irradiation (Figures 1C and S1A). We observed an upregulation of the complement system (represented in this panel only by C3) and the inflammation cascade at 4 and 24 hr, whereas both pathways were downregulated at 96 and 168 hr after irradiation (Figure 1C). Because 20 Gy might be of limited clinical relevance, we performed the same analysis 24 hr after irradiation with a single dose of 5 Gy and observed a similar transcriptional upregulation (Figure S1B).

C3 is the central protein of the complement cascade at which all three known pathways (classical, alternative, and lectin) converge and which gives rise to various bioactive components (Markiewski and Lambris, 2009). Because complement might be tumor-promoting (Markiewski et al., 2008; Markiewski and Lambris, 2009; Pio et al., 2014), we investigated whether radiotherapy-induced upregulation of complement supported or antagonized the efficacy of this treatment. We first quantified four different complement-related transcripts in response to radiotherapy: C3, C1s, Masp2 and Cfb. The classical and alternative pathways are the main pathways induced by radiotherapy on the transcriptional level (Figures 1D and S1C). Because NF-κB, JAK, and STAT transcriptional pathways (Chen et al., 2011; Fukuoka et al., 2013; Hasegawa et al., 2014; Huang et al., 2002), as well as S100 calcium-binding proteins A8 and A9 (S100A8, S100A9) (Schonthaler et al., 2013) are involved in the transcription of complement factors, we analyzed such pathways by immuno-blot and found an increased production of STAT 1, STAT 2, STAT 3, NF-κB, and JAK and increased phosphorylation of STAT 2, STAT3, and JAK (Figure S1D) 4 hr, but not



**Figure 2. C3a and C5a Are Crucial to the Therapeutic Efficacy of Radiotherapy**

(A) Control and  $C3^{-/-}$  mice were injected with B16F10-OVA cells and received radiotherapy 13 days later. Growth curves, ( $n = 5$  mice per group). Representative data from two independent experiments are shown. Radiotherapy, RT. (B) BALB/c,  $C3ar1^{-/-}$ ,  $C5ar1^{-/-}$ , and  $C3ar1^{-/-}C5ar1^{-/-}$  were injected with CT26 cells and received radiotherapy 13 days later. Tumor growth curves (BALB/c  $n = 5$  mice per group;  $C3ar1^{-/-}$ ,  $C5ar1^{-/-}$ , and  $C3ar1^{-/-}C5ar1^{-/-}$   $n = 8$  mice per group). Representative data from two independent experiments are shown. (C) Mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. SB290157 was administered at 2 mg/kg to block C3aR1 and anti-C5aR1 mAb 20/70 or an isotype control at 0.6 mg/kg to block C5aR1 (administered every second day starting on day 12 until day 19). ( $n = 5$  mice per group). Data are shown as the mean  $\pm$  SD. \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.00005$  by two-way ANOVA with the Bonferroni correction. See also Figure S2.

24 hr, after radiotherapy. Transcripts of *S100a8* and *S100a9* were unaffected (data not shown).

To show that these translated in different complement factor amounts, we analyzed the tumors prior to and after radiotherapy by immunofluorescence (Figure S1E) and by immuno-blot (Fig-

ure S1F). Because C1q and Factor B are more abundant compared to mannose-binding lectin C (MBL-C), these results confirmed our qPCR data (Figure S1C). We analyzed the activation status of complement and found deposition of fragments derived from C3 cleavage (C3b, iC3b, and C3c) 24 hr after radiotherapy in the proximity of (green fluorescence) or associated with (yellow fluorescence) blood vessels (Figure 1E). Similarly to C3 cleavage products, we observed C1q and Factor B mainly associated to blood vessels, whereas MBL-C showed a markedly different pattern of deposition. We obtained similar results using B16F10 cells, both at the transcriptional (data not shown) and protein level (Figure S1G). We confirmed radiotherapy-induced complement activation by immuno-blotting using an antibody recognizing C3 plus its fragments C3b, C3c, and C3dg (Figure S1H). Because deposition of C3b, iC3b, and C3c was not detected at other time points besides 24 hr after radiotherapy, this suggests that radiotherapy-induced complement activation is a rapid and transient response.

We analyzed the expression of complement-related transcripts and proteins in paired human tumors that were collected before and 24–36 hr after a single, low dose of radiotherapy (1.5 or 2 Gy) and detected upregulated expression (Figure 1F) and activation of complement (Figure 1G). To exclude that taking the first biopsy prior to radiotherapy rather than radiotherapy was responsible for complement activation, we analyzed additional four biopsies that were taken 24–36 hr after irradiation with 1.5–2 Gy but without previous intervention. The amounts of complement transcripts were comparable to those after radiotherapy in the paired samples, excluding this option (Figure 1F).

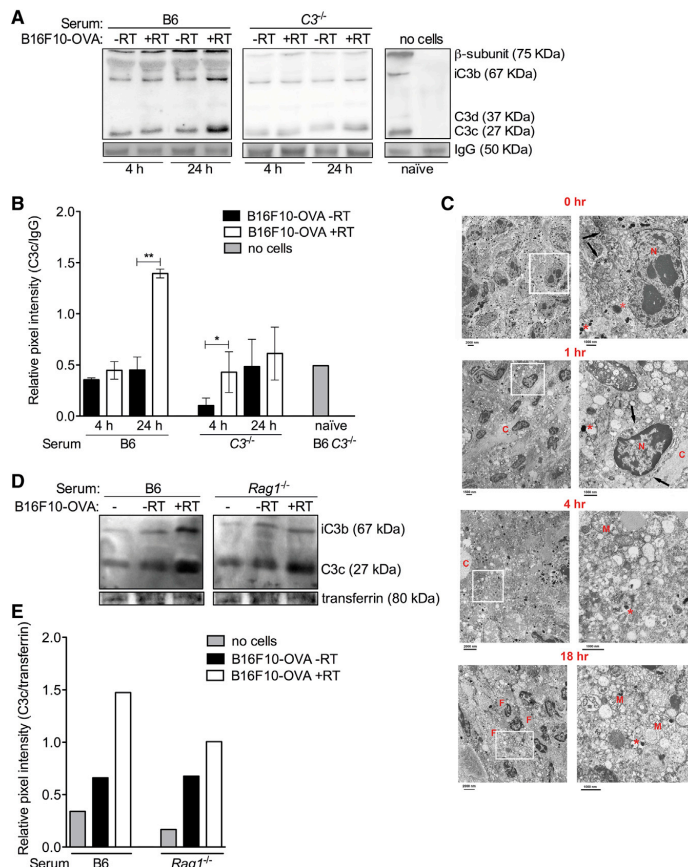
Together, we found that radiotherapy induces transient, local production, and activation of the classical and alternative complement pathway in both human and murine tumors.

### Therapeutic Efficacy of Radiotherapy Depends on C3a and C5a

To investigate the impact of complement activation on therapeutic efficacy, we applied radiotherapy to tumor-bearing  $C3^{-/-}$  mice. Because tumors grew more slowly in  $C3^{-/-}$  mice (Figures 2A and S2A) (Markiewski et al., 2008; Qing et al., 2012),  $C3^{-/-}$  and C57BL/6 mice were irradiated at two different time points, i.e., 13 (Figure 2A) or 17 days (Figure S2B) after tumor injection. Comparison of C57BL/6 mice irradiated at day 13 (Figure 2A, left panel) with  $C3^{-/-}$  mice irradiated at day 17 (Figure S2B, right panel) shows the response of tumors with a similar size (36–40 mm<sup>2</sup>) at the time point of irradiation. Radiotherapy was not efficient in  $C3^{-/-}$  mice irrespective of the day of therapy (Figures 2A, S2A, and S2B), suggesting that complement activation is crucial to efficacy.

Anaphylatoxins (C3a and C5a) modulate adaptive immunity (Schmudde et al., 2013; Strainic et al., 2013). As we observed higher local amounts of C3a and C5a (Figure S2C) and their receptors (Figures S2D) upon radiotherapy, we investigated their role in the response to radiotherapy using  $C3ar1^{-/-}$ ,  $C5ar1^{-/-}$ , and  $C3ar1^{-/-}C5ar1^{-/-}$  mice. As these mice were only available on a BALB/c background, we confirmed that radiotherapy resulted in local complement activation (Figure S2E), showing that radiotherapy-induced complement activation is a general phenomenon independent of the strain or tumor cell line used. Similar to  $C3^{-/-}$  mice,  $C5ar1^{-/-}$ ,





**Figure 3. Radiotherapy-Induced Tumor Cells Death Activates Complement**

(A and B) B16F10-OVA cells were irradiated in vitro with 20 Gy or left untreated. Serum from C57BL/6 or C3<sup>-/-</sup> mice was added to the cultures immediately after irradiation and C3 cleavage products were analyzed 4 and 24 hr later in the supernatants by immuno-blot using a polyclonal anti-C3 antibody (n = 3 mice per group). Representative data of two independent experiments are shown. (B) Relative intensities were calculated using Bio1D software. Radiotherapy, RT.

(C) Ultrastructural analysis of tumor response to radiotherapy. B16F10-OVA tumors were isolated from mice prior to and different times after radiotherapy. Fixed tumor tissue was epon embedded and analyzed by EM. Shown are images of tumor tissues before (0 h), 1, 4, and 18 hr after radiotherapy. Left column, overview; Right column, zoom-in of region indicated in left image. Bar indicates magnification. N, nucleus; M, mitochondrion; black arrow, cell boundaries; F, fat bodies; C, collagen; \*, melanosomes. (D and E) B16F10-OVA cells were irradiated in vitro with 20 Gy or left untreated. Serum from Rag1<sup>-/-</sup> mice was added to the cultures immediately after irradiation and C3 cleavage products were analyzed as described in (A), (n = mice 3 per group). (E) Relative intensities were calculated using Bio1D software.

Data in (B) are shown as the mean ± SD. \*p < 0.05, \*\*p < 0.005, by two-way ANOVA with the Bonferroni correction.

radiotherapy or a local tumor results in systemic activation of complement. Local radiotherapy results in apoptosis (Wang, 2008), mitotic catastrophe, and necrosis (Eriksson and Stigbrand, 2010), all of which can be potent activators of complement (Basu et al., 2000; Kemper et al.,

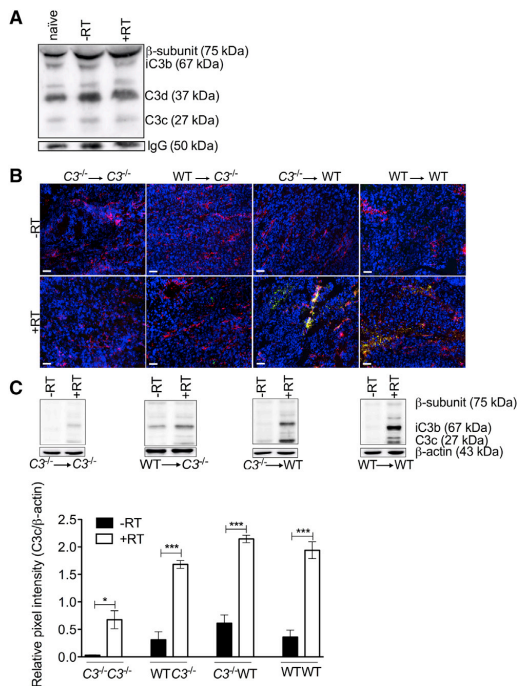
2008; Markiewski and Lambris, 2007). We first evaluated the possibility of direct complement activation by radiation and exposed serum from C57BL/6 or C3<sup>-/-</sup> mice in vitro to a single dose of 20 Gy or left them untreated and analyzed sera 4 and 24 hr after irradiation by immuno-blot. We did not detect any significant changes upon irradiation (data not shown). To investigate whether irradiated tumor cells can activate complement, we either or not exposed B16F10-OVA cells in vitro to a single dose of 20 Gy. Immediately after irradiation, 10-fold diluted serum from C57BL/6 mice or C3<sup>-/-</sup> mice was added to irradiated or untreated B16F10-OVA cells. Complement activation was detected in the supernatants 24 hr after radiation. Because the culture serum was from C3<sup>-/-</sup> mice, the complement should have been derived from the tumor cells (Figures 3A and 3B).

To identify the mode of tumor cell death upon a single dose of 20 Gy, we performed electron microscopy (EM) on B16F10-OVA tumors isolated at different time points after radiotherapy as indicated (Figure 3C). The tissue isolated before radiation showed normal nuclei, cell boundaries, intracellular organelles, and melanosomes (the dark vesicular structures) illustrating healthy

C3ar1<sup>-/-</sup>, and C3ar1<sup>-/-</sup>C5ar1<sup>-/-</sup> mice showed no significant impact of radiotherapy on tumor progression, whereas BALB/c mice did (Figures 2B, 2C, and S2F). To avoid the issue of inherent different tumor growth rates in various genetically ablated strains, we blocked C3aR with an antagonist (C3aRA, SB290157) or C5aR with a monoclonal antibody (20/70) (Baelder et al., 2005) just before applying radiotherapy to tumor-bearing C57BL/6 mice. This treatment blocked the improved antitumor effect of radiotherapy (Figures 2D and S2G), which is in line with the results observed in C3<sup>-/-</sup>, C3ar1<sup>-/-</sup>, C5ar1<sup>-/-</sup>, and C3ar1<sup>-/-</sup>C5ar1<sup>-/-</sup> mice. These data suggest that radiotherapy induces the intratumoral generation of anaphylatoxins, which are crucial to the therapeutic efficacy.

#### Radiotherapy-Induced Cell Death Locally Activates Complement

Despite the fact that complement deposition was observed in the vicinity of blood vessels, we failed to detect activated complement in serum irrespective of whether mice received radiotherapy or bore a tumor (Figure 4A). This excludes that



**Figure 4. Radiotherapy-Induced, Tumor-Associated Complement Is Mainly Liver-Derived but Partially Produced Locally**

(A) Measurement of complement activation by immuno-blot in pooled blood samples from naive, tumor-bearing, and irradiated tumor-bearing mice (24 hr after radiotherapy), (n = 3 mice per group). C3 cleavage products were detected using a polyclonal anti-C3 antibody. Radiotherapy, RT. (B and C) Bone-marrow chimeras were injected with B16F10-OVA cells and received radiotherapy 13 days later. Tumors were collected 24 hr after radiotherapy (B) Immunofluorescence of irradiated and untreated tumors. Sections were stained with an antibody recognizing C3b, iC3b, and C3c (green), CD31 for blood vessels (red) and DAPI (blue). Scale bars represent 100  $\mu$ m. Representative images from two independent experiments are shown (n = 4 mice per group). (C) C3 cleavage products in the tumors were analyzed by immuno-blot using a polyclonal anti-C3 antibody, (n = 3 mice per group). Relative expression measured by Bio-1D software. Representative data from two independent experiments are shown. Data in (C) are shown as the mean  $\pm$  SD. \*p < 0.05, \*\*\*p < 0.0005 by two-way ANOVA with the Bonferroni correction.

tumor tissue. Already 1 hr after radiation, the first signs of radiation damage were detected in the form of extensive vacuolization of tumor cells with normal nuclei and cell boundaries. Already 4 hr after radiation, cell boundaries were dissolved and cellular content fragmented. Mitochondrial structures (M) were swollen and did not show normal cristae. This did not change any further between 4 and 18 hr after radiotherapy. We did not observe any nuclear fragmentation and apoptotic bodies; rather, the irradiated tissue showed marked fields of necrosis with many intracellular materials now entering the extracellular space.

Because immunoglobulin M (IgM) binds to necrotic cells, which results in complement activation (Ciurana et al., 2004;

Quartier et al., 2005), we performed the experiment described above using serum from *Rag1*<sup>-/-</sup> mice. We observed reduced complement activation in supernatants containing *Rag1*<sup>-/-</sup> serum (Figures 3D and 3E), suggesting that IgM binding to necrotic cells contributes to radiotherapy-induced complement activation. This suggests that necrotic tumor cells express or secrete factors that activate complement.

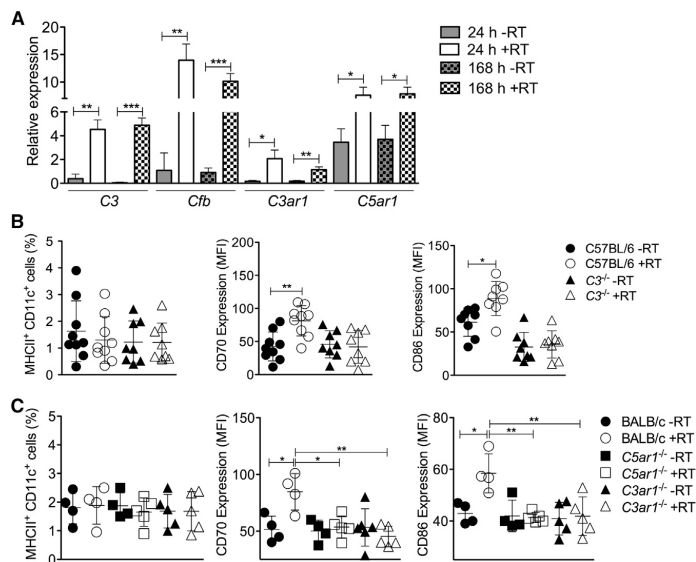
### Microenvironmental Complement Is Produced by Immune Cells

Hepatocytes are the main source of complement proteins, but also extra-hepatic tissues and immune cells can produce them (Farrar and Sacks, 2014; Kolev et al., 2014; Pio et al., 2014; Strainic et al., 2008). To define the source of radiotherapy-induced, tumor-associated complement, we generated *C3*<sup>-/-</sup>  $\rightarrow$  WT, WT  $\rightarrow$  *C3*<sup>-/-</sup>, WT  $\rightarrow$  WT, and *C3*<sup>-/-</sup>  $\rightarrow$  *C3*<sup>-/-</sup> bone marrow chimeras. B16F10-OVA bearing chimeras received radiotherapy or not and tumors were processed 24 hr later. We detected high amounts of activated complement in *C3*<sup>-/-</sup>  $\rightarrow$  WT and WT  $\rightarrow$  WT mice and less in WT  $\rightarrow$  *C3*<sup>-/-</sup> mice. The low amount of complement detected in irradiated tumors in *C3*<sup>-/-</sup>  $\rightarrow$  *C3*<sup>-/-</sup> mice is presumably tumor-derived (Figures 4B and 4C). This is in agreement with the detection of basal expression of complement-related transcripts in cultured B16F10-OVA tumor cells and an increased release of complement proteins by B16F10-OVA cells upon in vitro irradiation (Figures 3A, 3B, and 4C). These experiments suggest that a large fraction of radiotherapy-induced, tumor-associated complement is produced systemically, with a contribution of local production by immune and tumor cells.

### Radiotherapy-Induced DC Activation Depends on Anaphylatoxins

Because several immune cells can produce complement components (Li et al., 2007; Strainic et al., 2008), which are essential for full functional development of antigen-presenting cells (APCs) and T cell responses (Peng et al., 2009; Strainic et al., 2008), we investigated which immune cells produce complement or anaphylatoxin receptors in irradiated tumors. We sorted DCs, CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, and other CD45.2<sup>+</sup> cells from irradiated and untreated tumors (Figure S3A) and quantified the complement factor related transcripts *C3*, *C1s*, *Masp2*, *Cfb*, *C3ar1*, and *C5ar1* by qPCR. Because the impact of radiotherapy on tumor-associated DCs and T cells is apparent after 24 hr and 5–7 days, respectively (Gupta et al., 2012), we harvested tumors at these time points after radiotherapy exposure. DCs showed increased expression of *C3*, *Cfb*, *C3ar1*, and *C5ar1* 24 hr and 168 hr after radiotherapy (Figure 5A). We failed to detect expression of *C1s* and *Masp2* transcripts by DCs. The other CD45.2<sup>+</sup> cells (mainly containing macrophages) showed upregulated *C5ar1* but no other complement-related transcripts at 168 hr after radiotherapy (Figure S3B).

Recent studies demonstrate that complement factors, in particular anaphylatoxins, directly bind to their receptors on DCs thereby supporting their maturation, which then induces T cell effector activation (Li et al., 2007; Peng et al., 2009; Strainic et al., 2008). Furthermore, we showed previously that radiotherapy-induced activation of tumor-associated dendritic cells locally supports the function of tumor-specific CD8<sup>+</sup> T cells



**Figure 5. Radiotherapy Induces Upregulation of Transcripts for Complement and Anaphylatoxin Receptors by DCs and Is Essential to Their Maturation**

(A) Mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. DCs (CD45.2<sup>+</sup> TCRβ<sup>+</sup> CD11c<sup>+</sup> MHC-II<sup>high</sup> gated on live singlets) were sorted from untreated and irradiated tumors 24 and 168 hr after radiotherapy. Complement-related transcripts were quantified by qPCR (n = 5 mice per group). Radiotherapy, RT.

(B) Control and C3<sup>-/-</sup> mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. CD70 and CD86 expression on tumor-associated DCs in irradiated (48 hr after radiotherapy) and untreated tumors plotted as geometric mean fluorescence intensity (gMFI).

(C) BALB/c, C3ar1<sup>-/-</sup>, and C5ar1<sup>-/-</sup> mice were injected with CT26 cells and received radiotherapy 12 days later. DCs were analyzed as described in (B).

(B and C) Each symbol represents an individual mouse. Pooled data from two independent experiments are shown. Data are shown as the mean ± SD. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.0005 by two-way ANOVA with the Bonferroni correction. See also Figure S3.

and that this is crucial to therapeutic efficacy (Gupta et al., 2012). Therefore, we analyzed the activation status of DCs upon irradiation in C3<sup>-/-</sup> and in C57BL/6 mice 2 days after radiotherapy. DCs were equally present in untreated and irradiated tumors of complement-proficient and -deficient mice, however, DC activation as measured by surface expression of CD70 and CD86 (Keller et al., 2008) was observed only in irradiated C57BL/6, but not in C3<sup>-/-</sup> mice (Figure 5B). These results were confirmed using C3ar1<sup>-/-</sup>, C5ar1<sup>-/-</sup>, and BALB/c mice (Figure 5C). Thus, radiotherapy induces upregulation of anaphylatoxins, and their receptors in tumor-associated DCs controls radiotherapy-induced DC activation.

#### Radiotherapy-Induced CD8<sup>+</sup> T Cell Activation Depends on Anaphylatoxins

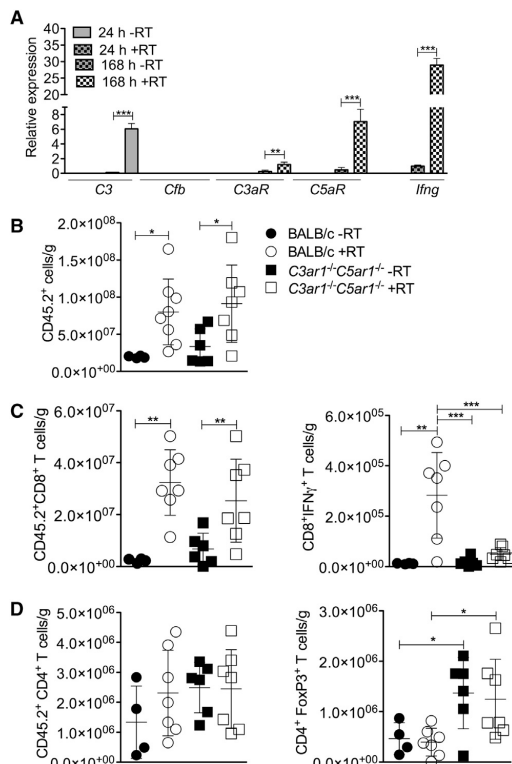
T cells express complement components and anaphylatoxin receptors that are critical to T cell co-stimulation (Liszewski et al., 2013; Strainic et al., 2008). CD8<sup>+</sup>, but not CD4<sup>+</sup> T, cells upregulated C3 and the anaphylatoxin receptors slowly at 168 hr, but not at 24 hr, after radiotherapy (Figures 6A and S3C), whereas C1s and Masp2 were undetectable. C5aR1 and C3aR1 signaling during cognate interaction between DCs and CD4<sup>+</sup> T cells promotes IFN-γ production (Liu et al., 2008) and counteracts the development of FoxP3<sup>+</sup> regulatory T cells (Strainic et al., 2013). We therefore analyzed the impact of radiotherapy on expression of *Ifng* and *Foxp3* and observed a strong upregulation of *Ifng* in CD8<sup>+</sup> T cells 168 hr after the treatment (Figure 6A), but no changes of *Foxp3* within the CD4<sup>+</sup> T cell compartment (data not shown).

To investigate the in vivo relevance of those observations, we analyzed the infiltrate in tumors from BALB/c and C3ar1<sup>-/-</sup>C5ar1<sup>-/-</sup> mice by flow cytometry 168 hr after radiotherapy. Radiotherapy resulted in higher numbers of CD45.2<sup>+</sup>

and CD8<sup>+</sup> T cells in tumors, which was independent of anaphylatoxins (Figure 6B). Radiotherapy had no effect on CD4<sup>+</sup> T cell numbers (Figures 6C and 6D). Therefore, it is unlikely that anaphylatoxins act as chemoattractants for T cells. We analyzed the global IFN-γ production by tumor-infiltrating CD8<sup>+</sup> T cells in situ. Therefore, we injected mice with Brefeldin A 4 hr before euthanasia and measured intracellular IFN-γ without in vitro restimulation. IFN-γ production by CD8<sup>+</sup> T cells was significantly increased in irradiated tumors in BALB/c, but not C3ar1<sup>-/-</sup>C5ar1<sup>-/-</sup> mice compared to untreated controls (Figures 6C, S4A, and S4B). This presumably involves anaphylatoxin-mediated DC maturation (Strainic et al., 2013), although a direct activity of anaphylatoxins on CD8<sup>+</sup> T cells cannot be excluded. In agreement with earlier data (Strainic et al., 2013), we observed higher numbers of FoxP3<sup>+</sup> T cells in C3ar1<sup>-/-</sup>C5ar1<sup>-/-</sup> mice independent of radiotherapy (Figures 6D, S4C, and S4D). Flow cytometry results thus confirmed the qPCR data on sorted cells (Figure 6A). We obtained similar results using B16F10-OVA-bearing C57BL/6 mice in which C3aR1 and C5aR1 were blocked (Figure S4E). A recent publication shows that blocking C3 improves the efficacy of fractionated radiotherapy given as multiple daily fractions of 1.5 Gy (Elvington et al., 2014), which is an apparent controversy with our findings. We found that 5 × 1.5 Gy equally diminished tumor growth as 1 × 20 Gy did. However, in contrast to a single high dose, 5 × 1.5 Gy resulted in prolonged activation of complement and did not support accumulation of CD8<sup>+</sup> T cells nor their function in the tumor (Figures S4G–S4I).

To generate a complete immune cell profile related to irradiation responses, we analyzed CD19<sup>+</sup> B cells and found low numbers (Figure S4F), regardless of radiotherapy or blockade of C5aR1 and C3aR1. Tumors contained substantial numbers of NK1.1<sup>+</sup> cells that significantly increased upon

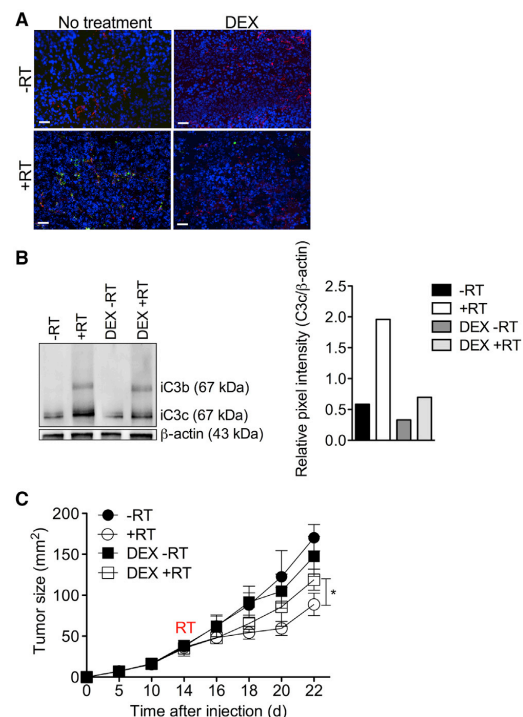




**Figure 6. Radiotherapy induces upregulation of transcripts for complement and anaphylatoxin receptors by CD8<sup>+</sup> T cells and supports tumor-specific immunity**

(A) C57BL/6 mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. CD8<sup>+</sup> T cells (CD45.2<sup>+</sup> TCRβ<sup>+</sup> CD8<sup>+</sup> gated on live singlets) were sorted from untreated and irradiated tumors 24 and 168 hr after radiotherapy. Complement-related transcripts were quantified by qPCR (n = 5 mice per group). Radiotherapy, RT. (B–D) BALB/c and C3ar1<sup>-/-</sup>C5ar1<sup>-/-</sup> mice were injected with CT26 cells and received radiotherapy 12 days later. Flow cytometric analysis was performed 7 days after radiotherapy. (B) The total number of infiltrating leukocytes (CD45.2<sup>+</sup>), (C) CD8<sup>+</sup> cells (left panel), and CD8<sup>+</sup> T cells that produce IFN-γ in vivo (right panel), (D) CD4<sup>+</sup> (left panel) and FoxP3<sup>+</sup> CD4<sup>+</sup> T cells (right panel) were determined per gram tumor tissue. Mice were injected with Brefeldin A 4 hr before euthanasia. Each symbol represents an individual mouse. Data are shown as the mean ± SD. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.0005 by two-way ANOVA with the Bonferroni correction. See also Figure S4.

radiotherapy and seemed independent of anaphylatoxin receptor blockade. We observed very low numbers of Nkp46<sup>+</sup> cells that remained similar in all four groups (Figure S4F). Thus, following a single dose of 20 Gy, the DC-CD8-arm of the immune system appears to be selectively activated in irradiated tumors, which is essential for tumor control and depends on complement.



**Figure 7. Dexamethasone inhibits complement activation and reduces the clinical efficacy of radiotherapy**

(A–C) Mice were injected with B16F10-OVA cells and received radiotherapy 14 days later. DEX (0.3 mg/kg) was injected daily from day 12 until day 22, (n = 6 mice per group). (A) Immunofluorescence of irradiated (24 hr) and untreated tumors. Sections were stained with an antibody recognizing C3b, iC3b, and C3c (green), CD31 for blood vessels (red), and DAPI (blue). Scale bars represent 100 μm. Representative images are shown. (B) C3 cleavage products in the tumors 24 hr after radiotherapy were analyzed by immuno-blot using a polyclonal anti-C3 antibody. Relative expression measured by Bio-1D software. Representative data are shown. (C) Tumor growth curves. The data are shown as the mean ± SD. \*p < 0.05 by two-way ANOVA with the Bonferroni correction. Radiotherapy, RT.

Dexamethasone (DEX) is a glucocorticoid with anti-inflammatory and immunosuppressive properties (Auphan et al., 1995). In addition, it inhibits the activation of complement (Engelman et al., 1995; Packard and Weiler, 1983). Given the important role of complement in promoting adaptive immunity and supporting the efficacy of radiotherapy, we treated mice with DEX starting 1 day before radiotherapy. DEX treatment significantly reduced the extent of local complement activation (Figure 7A) and importantly, also the efficacy of radiotherapy (Figures 7B and 7C).

Thus, radiotherapy-induced, local production of anaphylatoxins is essential to activation of DCs and protective effector function of CD8<sup>+</sup> T cells in the tumor and as such to therapeutic efficacy.

## DISCUSSION

In this study, we have demonstrated that radiotherapy induces an acute and transient local activation of complement, which is pivotal for tumor-specific immunity and therapeutic efficacy. Complement has traditionally been considered only to “complement” the action of the immune system in the antibody-mediated defense against pathogens. The current appreciation is that complement is involved in many different pathological processes such as transplant rejection, autoimmunity, neurodegeneration, and cancer.

The role of complement in cancer is still confusing as the production of complement-inhibiting proteins by tumor cells or stroma has been suggested to promote tumor growth (Kolev et al., 2011), whereas it is also proposed that complement in the context of chronic inflammation promotes tumor growth, migration and angiogenesis (Markiewski et al., 2008; Pio et al., 2014). This is in line with a previous publication (Elvington et al., 2014) showing increased efficacy of fractionated radiotherapy when C3 was blocked. Repeated irradiation might thus induce a chronic inflammatory response that interferes with protective adaptive immunity. In addition, the infiltrating T cells might be killed by the next dose before they could execute their anti-tumor effect. A different radiotherapy protocol that either introduces a radiotherapy holiday of 7–10 days between the fractions of radiotherapy or provides a single high-dose of radiotherapy might be required to optimally support tumor-specific immunity (Favaudon et al., 2014).

Pathogen- and damage-associated molecular patterns can activate C1q, MBL, and the alternative complement pathway. The latter can also be activated by spontaneous hydrolysis of C3 or by non-complement proteins (Markiewski and Lambris, 2007). Furthermore, modified membranes of late apoptotic and necrotic cells are potent activators of complement (Ricklin et al., 2010). In fact, every disturbance of homeostasis or assault might result in activation of complement (Kolev et al., 2014). Our data suggest that factors released from necrotic tumor cells upon radiotherapy are responsible for local complement activation. The leakiness of tumor-associated blood vessels (Carmeliet and Jain, 2011) might further promote accumulation of complement in the tumor.

The liver is the main source of complement, but many complement components can be produced by a variety of tissues and immune cells either constitutively or in response to stress (Kolev et al., 2014; Li et al., 2007). For example, locally produced C1q contributes to removal of apoptotic material and immune complexes (Roumenina et al., 2011) and supports T cell responses (Baudino et al., 2014). We have shown here that tumor-associated T cells, DCs, other CD45<sup>+</sup> cells, as well as tumor cells can be a source of anaphylatoxins and their receptors in response to radiotherapy. It is plausible to consider that tumor-associated stroma might also contribute to the production of complement upon radiotherapy.

Sensing immune cell-derived complement during cognate interactions between T cells and DCs is essential for development of protective immunity (Lalli et al., 2007; Liszewski et al., 2013; Peng et al., 2009; Strainic et al., 2008). Moreover, when signaling through C3aR1 and C5aR1 is prevented during cognate interactions, CD4<sup>+</sup> T cells develop into FoxP3<sup>+</sup> regulatory T (Treg) cells

instead of effectors (Strainic et al., 2013), in line with our observation that tumors contained more Treg cells in the absence of anaphylatoxin receptor signaling. We found that tumor-associated DCs produced complement factors and upregulated the expression of C3aR1 and C5aR1 upon radiotherapy, which appeared to be essential for radiotherapy-induced DCs maturation. It has been shown that anaphylatoxins directly can induce DC maturation in vitro and that C3 upregulation precedes the expression of IL-1, IL-12, and IL-23 (Strainic et al., 2008), suggesting a direct effect of anaphylatoxins on DCs. DC maturation is crucial to development and/or maintenance of T cell effector function within the tumor and efficacy of radiotherapy (Gupta et al., 2012), and indeed, tumor-infiltrating CD8<sup>+</sup> T cells failed to produce IFN- $\gamma$  after radiotherapy in the absence of signaling through C3aR1 and C5aR1.

Given the importance of complement activation and immune response following local radiotherapy, the administration of glucocorticoids, anti-inflammatory, and immunosuppressive drugs for managing post-radiation symptoms (Hempfen et al., 2002; Hughes et al., 2005) might have a modulating impact on the efficacy of radiotherapy. Indeed, DEX given around the time of radiotherapy significantly diminishes its efficacy, suggesting that treatment with glucocorticoids or other anti-inflammatory or immunosuppressive drugs might decrease the clinical response of cancer patients to radiotherapy.

The stimulation of tumor-specific immunity by standard therapies including radio- and chemotherapy has been documented in several publications (Formenti and Demaria, 2012; Gupta et al., 2012; Matsumura et al., 2008; Reits et al., 2006; Sharma et al., 2013) and this phenomenon might actually be of great clinical importance: dormant metastases are intrinsically resistant to standard treatments that mainly target rapidly dividing cells but might still be susceptible to immune-mediated control (Koebel et al., 2007). The abscopal effect—a situation in which not only the irradiated tumor but also distant lesions show a clinical response—can be explained as such. When radiotherapy is combined with immune stimulation by anti-CTLA-4 antibodies, the abscopal effect becomes readily apparent (Postow et al., 2012; Verbrugge et al., 2014).

Our data expand the role of complement in the defense against tumors. Tumor-specific immunity is unleashed by locally produced anaphylatoxins in response to radiotherapy that activate DCs and then CD8<sup>+</sup> T cells for optimal tumor control following radiotherapy.

## EXPERIMENTAL PROCEDURES

### Mice and Cell Lines

C57BL/6, C3<sup>−/−</sup>, BALB/cJ, C5aR1<sup>−/−</sup>, C3aR1<sup>−/−</sup>, and Rag1<sup>−/−</sup> mice were purchased from the Jackson Laboratory. C3<sup>−/−</sup> mice were on a C57BL/6.129S4 background; C3aR1<sup>−/−</sup> and C5aR1<sup>−/−</sup> mice on a BALB/cJ background, Rag1<sup>−/−</sup> mice were on a C57BL/6 background. We generated C5aR1<sup>−/−</sup> C3aR1<sup>−/−</sup> mice by crossing C5aR1<sup>−/−</sup> and C3aR1<sup>−/−</sup> mice. All mice were bred and maintained in specific pathogen-free facilities at the University of Zurich and University Hospital of Zurich. C57BL/6 → C3<sup>−/−</sup>, C3<sup>−/−</sup> → C57BL/6, C3<sup>−/−</sup> → C3<sup>−/−</sup>, and C57BL/6 → C57BL/6 bone-marrow chimeras were generated as previously described (Probst et al., 2003). All experiments were performed with age- and sex-matched mice in accordance with the guidelines of the Swiss federal and cantonal laws on animal protection.

B16F10 melanoma cells (ATCC) and B16F10-OVA (B16F10 stably transfected to express chicken ovalbumin as neo-antigen, provided by Melody

Swartz, EPFL) were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen), CT26 murine colon carcinoma (ATCC) cells in MEM medium (Invitrogen). Media were supplemented with 10% fetal calf serum,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10 mM sodium pyruvate 2 mM L-glutamine, and antibiotics.

#### In Vivo Experiments

$2 \times 10^5$  B16F10-OVA, B16F10 or CT26 were injected s.c. in 100  $\mu$ l of a 1:1 mix of PBS and Matrigel Basement Membrane Matrix (BD Biosciences).

Local radiotherapy with a single dose of 20 or 5 Gy was performed at indicated time points using a Xstrahl 200 kV X-ray unit at 1 Gy/min as described (Gupta et al., 2012). Prior to radiotherapy, mice were anaesthetized by i.p. injection of 50 mg/kg ketamine and 10 mg/kg xylazine.

The C3aR1 antagonist (C3aRA) N2-[(2,2-Diphenylethoxy)acetyl]-L-arginine (SB290157, Calbiochem) was administered i.p. at 2 mg/kg body weight in 100  $\mu$ l PBS. Anti-C5aR1 mAb 20/70 (Hycult Biotech) or an isotype control (rat IgG2b anti-HLA-DR, clone SFRF8B6) was administered i.p. at 0.6 mg/kg in 100  $\mu$ l PBS (Baelder et al., 2005; Godau et al., 2004; Sun et al., 2011). C3aRA and anti-C5aR1 mAb were administered every second day starting at the day of radiotherapy until the end of the experiment.

To measure the cytokine production in vivo, we injected Brefeldin A (BFA, Sigma-Aldrich). A 20-mg/ml stock was prepared in DMSO. Further dilution to 0.5 mg/ml was made in PBS, and 500  $\mu$ l was injected i.p. 4 hr before mice were sacrificed (Liu and Whitton, 2005). DEX (Sigma-Aldrich) was administered daily at 0.3 mg/kg in PBS p.o., starting 1 day before radiotherapy. Tumors were measured with a caliper every 2–3 days in two dimensions (length and width). Excised tumors were weighed and processed for flow cytometry, histology, or isolation of RNA and proteins.

#### In Vitro Experiments

C57BL/6 and C3<sup>-/-</sup> mice were bled from the sublingual vein and sera were collected with Microtainer SST tubes (BD). Sera were diluted 1:10 with PBS, irradiated with 20 Gy (YXLON Y.SMART582, YXLON International GmbH, Hamburg, D) and subsequently incubated at 37°C. B16F10-OVA cells were cultured in 6-well plates ( $10^5$  cells per well) and irradiated as described above. Immediately after irradiation, 60  $\mu$ l of serum from C57BL/6 or C3<sup>-/-</sup> mice diluted in 500  $\mu$ l of PBS was added to the cells. Control samples were not irradiated but treated similarly otherwise. Supernatants were taken 4 hr and 24 hr after irradiation and were stored at  $-80^\circ\text{C}$  until further use.

#### Human Samples

Two sets of biopsies were provided by the Department of Dermatology, University Hospital Zurich. Patients signed informed consent. The ethical committee of the canton of Zurich approved this study (EK647). (1) Paired biopsies were collected from two patients with basal cell carcinoma and one with lentigo maligna melanoma immediately before and 24–36 hr after radiotherapy (1.5–2 Gy) and were snap frozen. (2) Four biopsies were collected from four patients (two with basal cell carcinoma, one with melanoma, and one with squamous cell carcinoma) 24–36 hr after radiotherapy (1.5–2 Gy) but without previous interventions and were snap frozen.

#### Statistical Analysis

Results are presented as mean  $\pm$  SD. Statistical significance was determined by ANOVA using GraphPad Prism 5 software (GraphPad). When multiple groups were compared, we used the Bonferroni post-test correction. When two groups were compared, we used the two-tailed Student's t test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.03.009>.

#### AUTHOR CONTRIBUTIONS

L.S., A.K., A.G., and M.v.d.B. conceptualized the study and designed experiments; L.S. conducted the majority of the experiments; V.L. and A.B. per-

formed immuno-blot experiments; M.O. created heatmaps and performed bioinformatics analyses; A.O.F. and M.P. irradiated mice; V.C. sorted cells and performed some experiments; R.D. provided patient biopsies; H.J. and J.N. performed electron microscopy; and L.S. and M.v.d.B. wrote the manuscript.

#### ACKNOWLEDGMENTS

We thank Melody Swartz (EPFL Lausanne, Switzerland) for providing the B16F10-OVA cell line; Jovan Pavlovic (Institute of Medical Virology, University of Zurich) for providing antibodies against JAK and STAT; James Di Santo (Department of Immunology, Institute Pasteur, Paris) for providing anti-NK1.1 antibody; Claudia Matter (Clinic for Oncology, University Hospital Zurich, Switzerland) and Karina Silina (Institute of Experimental Immunology, University of Zurich) for technical assistance; personnel from the Biologisches Zentrallabor (University Hospital of Zurich) for expert animal care; Alessandra Curioni-Fontecedro (Clinic for Oncology, University Hospital of Zurich) for helpful discussion; and Burkhard Becher, Christian Münz, Ali Bransi (Institute of Experimental Immunology, University of Zurich), Sabine Werner (Institute of Cell Biology, ETH, Zurich), Stefano Ferrari (Institute of Molecular Cancer Research, University of Zurich), Mark Robert Nicolls (Department of Medicine, Stanford Medicine), and John Lambris (Department of Pathology, University of Pennsylvania) for scientific support and critically reading the manuscript. This work was financially supported by the Swiss National Science Foundation (SNSF), the Vontobel Foundation Zurich, and the University Research Priority Program (URPP) "Translational Cancer Research."

Received: July 1, 2014

Revised: January 16, 2015

Accepted: March 21, 2015

Published: April 14, 2015

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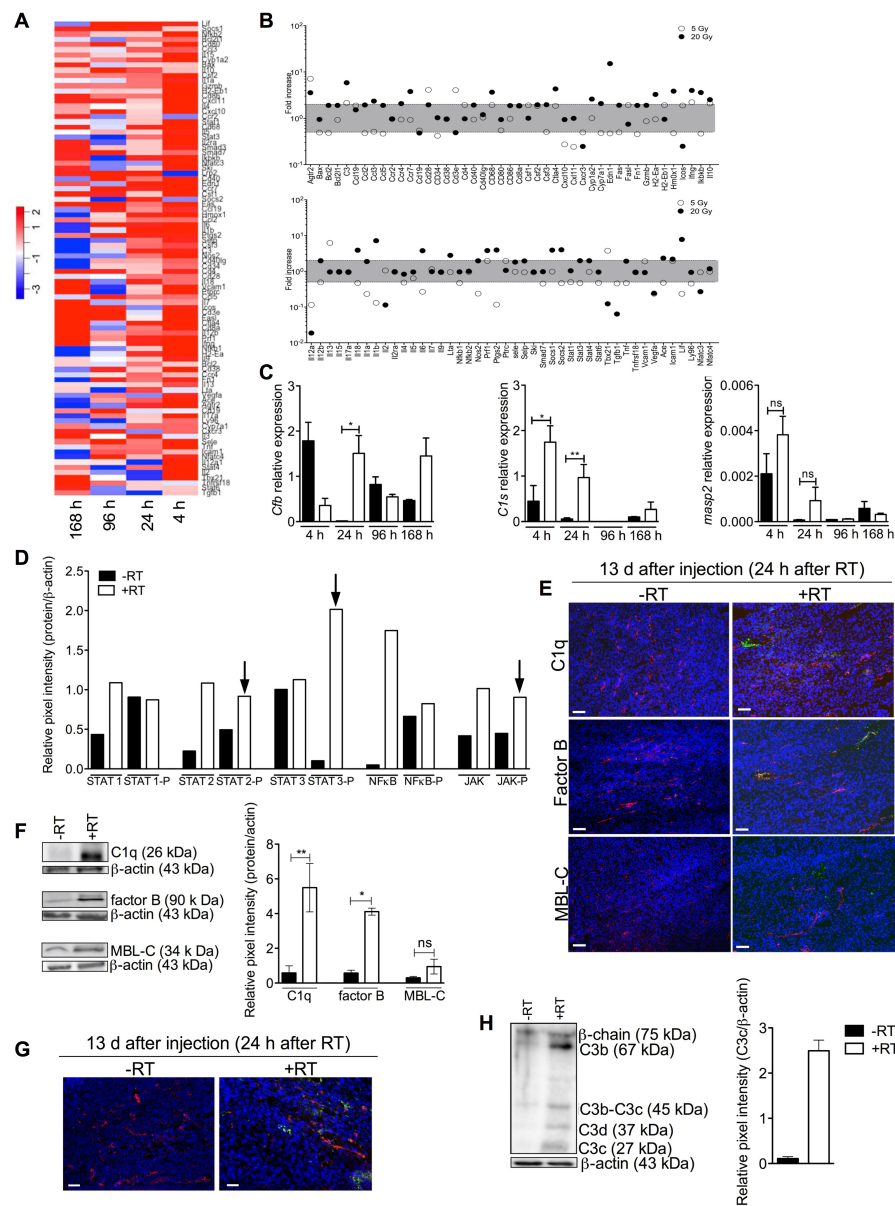
Immunity

Supplemental Information

**Complement Is a Central Mediator  
of Radiotherapy-Induced Tumor-Specific  
Immunity and Clinical Response**

Laura Surace, Veronika Lysenko, Andrea Orlando Fontana, Virginia Cecconi, Hans Janssen, Antonela Bicvic, Michal Okoniewski, Martin Pruschy, Reinhard Dummer, Jacques Neefjes, Alexander Knuth, Anurag Gupta, and Maries van den Broek





**Figure S1. Radiotherapy results in transient upregulation and activation of complement in tumors. Related to Figure 1.**

(A) Radiotherapy-induced changes in immune response-related transcripts. Heatmaps of transcripts were created using the log<sub>2</sub> value of the fold change of irradiated compared to untreated tumors at different time points. (*n* = 6 mice per time point).

(B) Comparison of immune response-related transcripts after a single dose of 5 Gy or 20 Gy. Values represent the fold-increase of irradiated vs untreated samples. Open symbols, 5 Gy; closed symbols, 20 Gy. Grey area, no changes in the irradiated sample

compared to the untreated one ( $n = 6$  mice per group).

(C) Radiotherapy activates the classical and alternative pathway of complement. C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated 12 d later. Relative expression of *Cfb* (alternative pathway), *C1s* (classical pathway) and *Masp2* (lectine pathway) transcripts at indicated time points after radiotherapy. The data shown are the mean  $\pm$  SD of triplicates from three independent analyses.

(D) Increased phosphorylation of STAT 2, STAT 3 and JAK 4 h after radiotherapy. C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated 12 d later. Measurement of STAT 1, STAT 2, STAT 3, NF- $\kappa$ B, JAK and their phosphorylated forms by immuno-blot in pooled blood samples from tumor-bearing and irradiated tumor-bearing mice (4 h after radiotherapy), ( $n = 3$  per group).

(E) Immunofluorescence staining for C1q (classical pathway), Factor B (alternative pathway) and MBL-C (lectine pathway) in irradiated (24 h) and untreated tumors. ( $n = 6$  mice per group).

(F) Quantification of C1q, Factor B and MBL-C by immuno-blot. A representative figures from two different experiments is shown.

The data (C) are shown as the mean  $\pm$  SD.  $*P < 0.05$ ,  $**P < 0.005$  by two-way analysis of variance (ANOVA) with the Bonferroni correction.

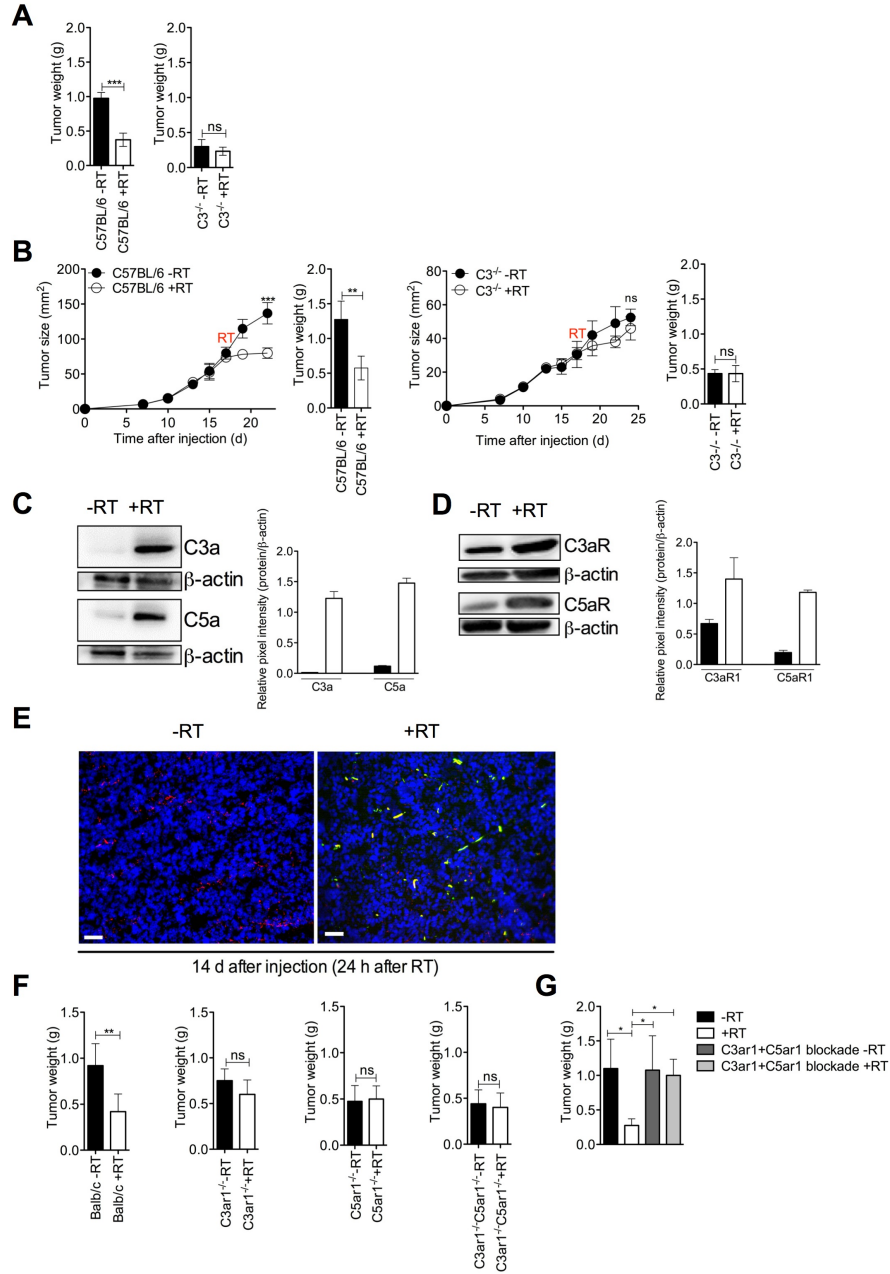
(G) Radiotherapy-induced complement activation in B16F10 tumors. C56BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10 cells and tumors were irradiated 13 d later. Sections were stained with antibodies recognizing C3b, iC3b and C3c (green), CD31 for blood vessels (red) and DAPI (blue). Scale bars, 100 $\mu$ m.

(H) Complement is activated in tumors 24 h after radiotherapy. C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated 12 d later. Immuno-blot for C3 cleavage products detected with a polyclonal anti-C3 antibody.

(D,F,H) Relative band intensities were measured using the Bio-1D software. Representative data from three independent experiments are shown.

(C,D,F,H) Open bars, irradiated (+RT); filled bars, untreated (-RT).





**Figure S2. C3a and C5a are crucial to the therapeutic efficacy of radiotherapy. Related to Figure 2.**

(A,B) C3<sup>-/-</sup> mice do not respond to radiotherapy. C57BL/6 and C3<sup>-/-</sup> mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated (RT) either 13 d or 17 d later. ( $n = 5$  mice per group). (A) Tumor weight at endpoint. (B)

B16F10-OVA tumors irradiated 17 d after tumor injection. Tumor growth curve and weight at endpoint. Representative data from two independent experiments are shown as the mean  $\pm$  SD.  $**P < 0.005$ ,  $***P < 0.0005$  by Student's t test.

(C) Radiotherapy results in increased expression of C3a, C5a and their receptors in the tumor. C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated 12 d later. Detection of C3a and C5a in the untreated and irradiated tumors by immuno-blot.

(D) Detection of C3aR1 and C5aR1 in irradiated (24 h) and untreated tumors analyzed by Western blot. Representative data from two independent experiments are shown.

(E) Radiotherapy-induced complement activation in CT26 tumors. BALB/c mice were injected s.c. with  $2 \times 10^5$  CT26 cells and tumors were irradiated 14 d later. Sections were stained with antibodies recognizing C3b, iC3b and C3c (green), CD31 for blood vessels (red) and DAPI (blue). Scale bars, 100 $\mu$ m.

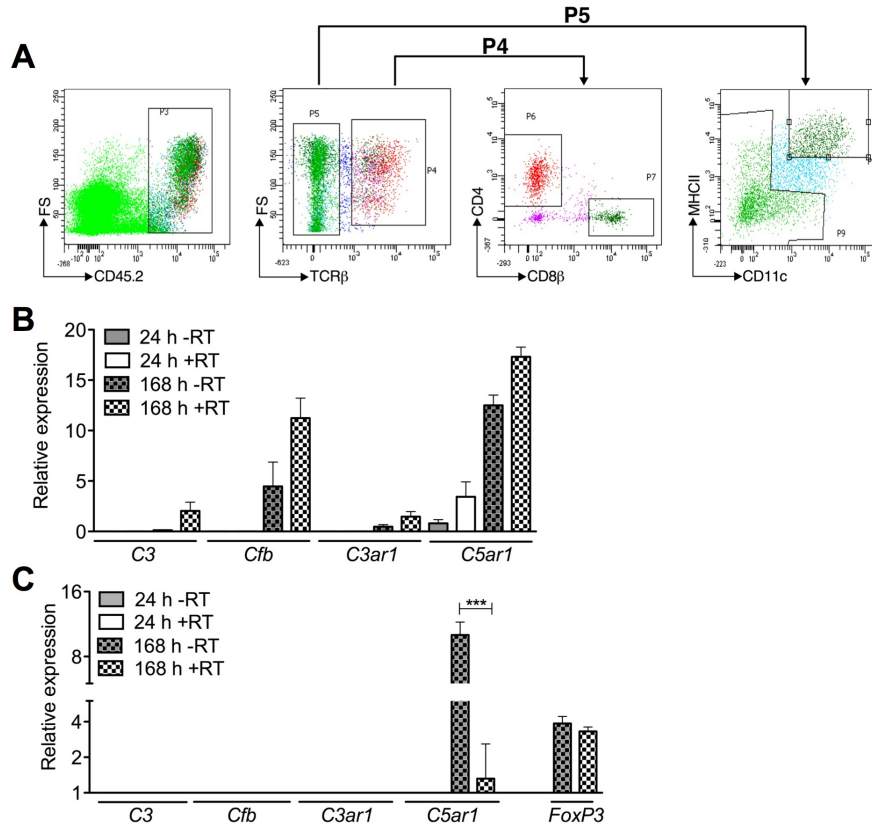
(F) C3a and C5a are key players in radiotherapy response. BALB/c, *C3ar1*<sup>-/-</sup>, *C5ar1*<sup>-/-</sup> and *C3ar1*<sup>-/-</sup>*C5ar1*<sup>-/-</sup> mice were injected s.c. with  $2 \times 10^5$  CT26 cells and tumors were irradiated 13 d later. Weight of tumors at endpoint. BALB/c:  $n = 5$  mice per group; *C3ar1*<sup>-/-</sup>, *C5ar1*<sup>-/-</sup> and *C3ar1*<sup>-/-</sup>*C5ar1*<sup>-/-</sup>:  $n = 8$  mice per group.

(G) C3a and C5a are key players in radiotherapy response. C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated 12 d later. To block the C3aR1, the C3aR1 antagonist SB290157 was administered i.p. at 2 mg/kg body weight in 100  $\mu$ l PBS. To block C5aR1, animals were treated with the neutralizing anti-C5aR1 mAb 20/70 or an isotype control i.p. at 0.6 mg/kg in 100  $\mu$ l PBS. C3aRA and anti-C5aR1 mAb were administered every second day starting at the day of radiotherapy until the end of the experiment. Weight of tumors at endpoint.  $n = 5$  mice per group.

The data are shown as the mean  $\pm$  SD.  $*P < 0.05$ ,  $**P < 0.005$  by Student's t test (A) and two-way analysis of variance (ANOVA) with the Bonferroni correction (B).

(C,D) Relative pixel intensities were calculated using Bio-1D software.

(A-D,F) Open bars, irradiated (+RT); filled bars, untreated (-RT).



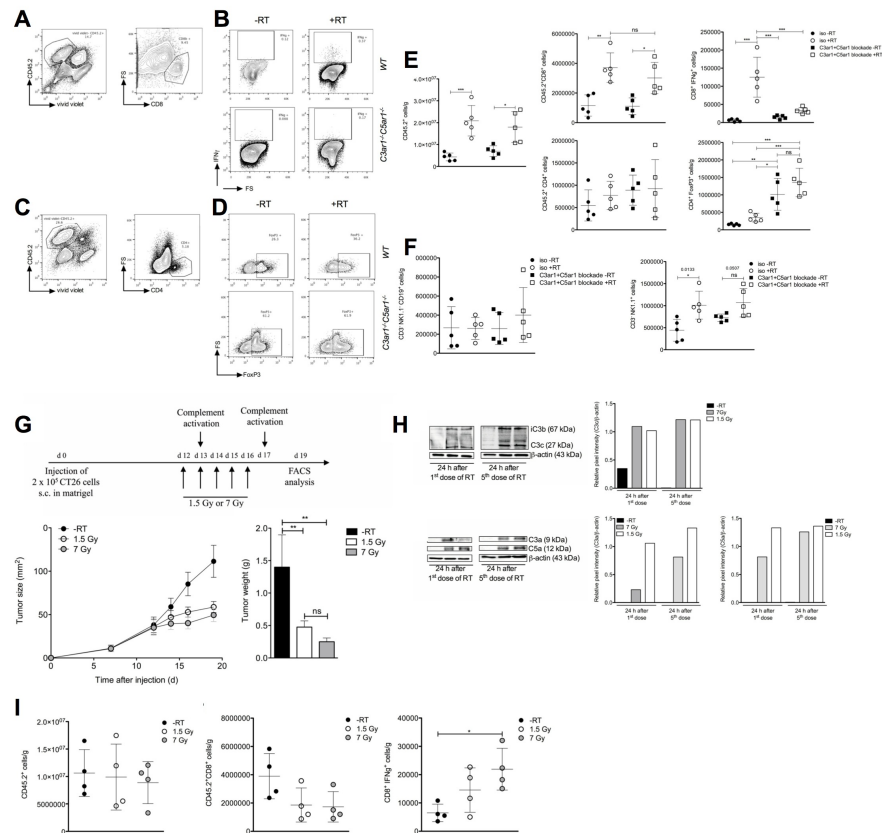
**Figure S3. Radiotherapy induces upregulation of transcripts for complement and anaphylatoxins receptors by immune cells. Related to Figure 5.**

C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated 12 d later ( $n = 5$  mice per group).

(A) CD8<sup>+</sup> T cells (P7, CD45.2<sup>+</sup> TCRβ<sup>+</sup> CD8<sup>+</sup>), CD4<sup>+</sup> T cells (P6, CD45.2<sup>+</sup> TCRβ<sup>+</sup> CD4<sup>+</sup>), DCs (P8, CD45.2<sup>+</sup> CD11c<sup>+</sup> MHCII<sup>high</sup>) and the rest of CD45.2<sup>+</sup> cells (P9) were sorted from untreated and irradiated tumors. Samples were gated on live singlets.

(B) CD45.2<sup>+</sup> cells and (C) CD4<sup>+</sup> T cells were sorted from untreated and irradiated tumors 24 and 168 h after radiotherapy. Complement-related transcripts were quantified by qPCR.

Data are shown as the mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  by two-way analysis of variance (ANOVA) with the Bonferroni correction.



**Figure S4. Radiotherapy fails to promote IFN- $\gamma$  production in *C3ar1*<sup>-/-</sup>*C5ar1*<sup>-/-</sup> mice; a higher number of FoxP3<sup>+</sup> cells in both untreated and irradiated tumors in *C3ar1*<sup>-/-</sup>*C5ar1*<sup>-/-</sup> mice. Related to Figure 6.**

BALB/c and *C3ar1*<sup>-/-</sup>*C5ar1*<sup>-/-</sup> mice were injected s.c. with  $2 \times 10^5$  CT26 cells and tumors were irradiated 13 d later. Mice were injected with Brefeldin A 4 h before euthanasia followed by immediate intracellular staining for IFN- $\gamma$ .

(A) Gating strategy for CD8<sup>+</sup> T cells.

(B) Representative example of IFN- $\gamma$  production by CD8<sup>+</sup> T cells in irradiated and untreated tumors in BALB/c or *C3ar1*<sup>-/-</sup>*C5ar1*<sup>-/-</sup> mice.

(C) Gating strategy for CD4<sup>+</sup> T cells.

(D) Representative example of FoxP3 expression by CD4<sup>+</sup> T cells in irradiated and untreated tumors in BALB/c or *C3ar1*<sup>-/-</sup>*C5ar1*<sup>-/-</sup> mice.

(E,F) C3aR1 and C5aR1 blockade prevents the production of IFN- $\gamma$  by CD8<sup>+</sup> T cells, increases the number of Treg, but it does not affect NK cells and B cells numbers. C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10-OVA cells and tumors were irradiated (+RT) on d 12. To block the C3aR1, the C3aR antagonist SB290157 was administered i.p. at 2 mg/kg body weight in 100  $\mu$ l PBS. To block C5aR1, animals were treated with the neutralizing anti-C5aR1 mAb 20/70 or an isotype control i.p. at

0.6 mg/kg in 100  $\mu$ l PBS. C3aRA and anti-C5aR1 mAb were administered every second day starting at the day of radiotherapy until the end of the experiment. ( $n = 5$  mice per group). (E) *Left panel*: The total number of infiltrating leukocytes (CD45.2<sup>+</sup>) per gram tumor tissue. *Upper right panels*: The total number of CD8<sup>+</sup> cells (CD45.2<sup>+</sup> CD8<sup>+</sup>, left) and of CD8<sup>+</sup> cells that produce IFN- $\gamma$  *in vivo* (right) per gram tumor tissue. *Lower right panels*: The total number of CD4<sup>+</sup> (CD45.2<sup>+</sup> CD4<sup>+</sup>, left) and of FoxP3<sup>+</sup> CD4<sup>+</sup> cells (right) per gram tumor tissue. (F) *Left panel*: The total number of B cells (CD3<sup>-</sup>NK1.1<sup>-</sup>CD19<sup>+</sup>) per gram tumor tissue. *Middle panel*: The total number of NK cells (CD3<sup>-</sup>NK1.1<sup>+</sup>) cells per gram tumor tissue. *Right panel*: The total number of CD3<sup>-</sup>NK1.1<sup>+</sup>Nkp46<sup>+</sup> cells per gram tumor tissue.

Cells were gated on live singlets, every symbol represents an individual mouse.

(G-I) Fractionated irradiation causes chronic complement activation and compromises CD8<sup>+</sup> T cell infiltration.

(G) C57BL/6 mice were injected with  $2 \times 10^5$  B16F10-OVA cells and treated with daily fractions of 1.5 Gy or 7 Gy (to reach a cumulative dose of 20 Gy) for 5 consecutive days starting on d 12. Mice were euthanized on d 13 (24 h after the first fraction of radiation), d 17 (24 h after the last fraction of radiation) and d 19 (7 d after starting therapy). Fractionated treatments delayed tumor-progression. Growth curves of B16F10-OVA tumors ( $n = 4$  mice per group). Untreated (closed black circles), irradiated with 1.5 Gy (open circles) and irradiated with 7 Gy (closed grey circles). Tumor weight at endpoint (d 19).

(H) Each irradiation results in complement activation and production of anaphylatoxins. Quantification of C3 cleavage products and (D) anaphylatoxins (C3a, C5a) by immuno-blot 24 h after the first and the last dose of radiotherapy (1.5 or 7 Gy). Every dose resulted in complement activation and production of anaphylatoxins. Relative band intensities were calculated by using Bio-1D software, ( $n = 2$  mice).

(I) Fractionated radiotherapy does not result in higher infiltration by leukocytes. The total number of leukocytes (CD45.2<sup>+</sup> cells of live singlets) per gram tumor tissue. Fractionated radiotherapy did not result in higher infiltration by CD8<sup>+</sup> T cells. Although the number of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells was significantly higher in the group treated with 5x 7 Gy when compared to control mice, this was not the case after treatment with 5x 1.5 Gy. The total number of CD8<sup>+</sup> cells (CD45.2<sup>+</sup> CD8<sup>+</sup>, left panel) and of CD8<sup>+</sup> cells that produce IFN- $\gamma$  *in vivo* (right panel) per gram tumor tissue. Mice were injected i.p. with Brefeldin A 4 h before euthanasia followed by immediate intracellular staining for IFN- $\gamma$ . Fractionated radiotherapy did not result in higher infiltration by CD8<sup>+</sup> T cells. Although the number of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells was significantly higher in the group treated with 5x 7 Gy when compared to control mice, this was not the case after treatment with 5x 1.5 Gy. Every symbol represents an individual mouse.

Data are shown as the mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  by two-way analysis of variance (ANOVA) with the Bonferroni correction.

+RT, irradiated; -RT, untreated.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Quantitative real-time PCR (qPCR)

Tumors were harvested 4, 24, 96 and 168 h after radiotherapy. For each time point, untreated tumors were used as control. Excised B16F10-OVA tumors were collected in 1 mL Trizol Reagent (Life Technologies) per 0.2 g of tissue. Samples were homogenized with the FastPrep24 homogenizer (MpBio) for 2 cycles of 30" according to the manufacturer's instructions and RNA was isolated according to the Trizol protocol. The extracted RNAs from three tumors of the same experimental group were pooled. The concentration and the purity of the extracted RNA were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with a RIN (RNA integrity number) > 3 were used for subsequent qPCR. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. qPCR was performed using commercially available TaqMan primers (Applied Biosystems). Reactions were run on the RotoGene Cyclor (Corbett Research). Results were normalized to *Gadph* and displayed as relative expression.

### TaqMan mouse immune response array

Samples were processed and RNA was extracted and reverse transcribed as described above and only samples with a RIN > 5 were used. cDNA was used to run the TaqMan Array 96-well Mouse Immune Response Plate in a AB 7900HT (Applied Biosystems) machine according to the manufacturer's instructions. Data were collected as CT values and log<sub>2</sub> of the fold change values comparing tumors with and without radiotherapy were calculated. These values were normalized against the four different endogenous controls present in the panel meaning (*Gadph*, *Hprt1*, *Gusb* and *18S*). Data were processed using R/Bioconductor (Gentleman et al., 2004). The heatmap was generated with the heatmap.2 function from the package gplots and represents the clustering of samples using the Pearson's correlation as a distance measure and "complete" linkage function. Genes for the plot of the time series have been selected by a formula choosing shape of the profile in the dataset where log<sub>2</sub> fold changes in the time point behave as follows: FC<sub>t1</sub>>0 FC<sub>t2</sub>>0 and FC<sub>t4</sub><0. The selected genes are coloured according to the log<sub>2</sub> fold change values at t2 from highest (red) to lowest (blue).

### Western blotting

Tumors were collected in PBS containing a cocktail of protease inhibitors (SIGMAFAST protease inhibitor, Sigma-Aldrich) prepared as indicated by the provider. Serum was collected with Microtainer SST tubes (BD) at the indicated time points to check for systemic complement activation. Tumors were homogenized with the FastPrep24 homogenizer as described above. The protein concentration in homogenized tumor samples was determined using a NanoDrop ND-1000 Spectrophotometer or by a Bradford assay (Promega). Western blots were performed as described (Lin et al., 2001) using 12% polyacrylamide gels. Blots were evaluated

using a Fusion-FX7 SPECTRA machine and Bio1D software (VILBER LOURMAT, Eberhardzell, Germany). Results are expressed as relative pixel intensity, which was calculated as follows: pixels of the protein of interest divided by the pixels of the internal standard of the same sample ( $\beta$ -actin for tissues, transferrin or IgG for serum).

### **Immunofluorescence**

Tumors were collected in OCT (Tissue-Tek Optimal Cutting Temperature Compound, Sakura), gently frozen in liquid nitrogen and stored at -80°C for 24 h. Samples were then left for 30' at -20°C and processed into 7- $\mu$ m thick sections using a microtome at -20°C. Cryosections were blocked for 1 h at room temperature in a blocking solution (PBS containing 10% FCS and 0.1% TritonX), washed with PBS and incubated overnight at 4°C with primary antibodies diluted in blocking solution. Sections were washed with PBS and incubated with secondary antibodies diluted in blocking solution for 1 h at room temperature. After a final wash with PBS, slides were mounted in Mowiol anti-fade solution (Sigma-Aldrich). Sections were photographed using a Leica DMI6000 inverted microscope (Leica microsystem, Wetzlar, Germany). Sections were evaluated using ImageJ software.

### **Quantitative and qualitative analysis of complement activation**

To quantitatively and qualitatively analyze the presence of proteins of the complement system in the tumor mass, we performed Western blot analyses and immunofluorescence.

For Western blot analysis, tumors were collected in PBS containing a cocktail of protease inhibitors (SIGMAFAST protease inhibitor, Sigma-Aldrich) prepared as indicated by the provider. Serum was collected with Microtainer SST tubes (BD) at the indicated time points to check for systemic complement activation. Tumors were homogenized with the FastPrep24 homogenizer as described above. The protein concentration in homogenized tumor samples was determined using a NanoDrop ND-1000 Spectrophotometer or by a Bradford assay (Promega). Western blots were performed as described (Lin et al., 2001) using 12% polyacrylamide gels. Blots were evaluated using a Fusion-FX7 SPECTRA machine and Bio1D software (VILBER LOURMAT, Eberhardzell, Germany).

For immunofluorescence, tumors were collected in OCT (Tissue-Tek Optimal Cutting Temperature Compound, Sakura), gently frozen in liquid nitrogen and stored at -80°C for 24 h. Samples were then left for 30' at -20°C and processed into 7- $\mu$ m thick sections using a microtome at -20°C. Cryosections were blocked for 1 h at room temperature in a blocking solution (PBS containing 10% FCS and 0.1% TritonX), washed with PBS and incubated overnight at 4°C with primary antibodies diluted in blocking solution. Sections were washed with PBS and incubated with secondary antibodies diluted in blocking solution for 1 h at room temperature. After a final wash with PBS, slides were mounted in Mowiol anti-fade solution (Sigma-Aldrich). Sections were photographed using a Leica DMI6000 inverted microscope (Leica microsystem, Wetzlar, Germany). Sections were evaluated using ImageJ software.

Activated complement was detected using rat anti-mouse C3b/iC3b/C3c mAb (clone 2/11, Hycult Biotech) that specifically recognizes C3 cleavage products but not inactive C3 (Mastellos et al., 2004). Furthermore, we used rat anti-mouse C1q mAb (Abcam), goat anti-mouse MBL (Santa Cruz Biotechnology), rabbit anti-mouse Factor B (Thermo Fisher Scientific), polyclonal rabbit anti-mouse C3 (Hycult Biotech), rat anti-mouse C3a (clone 3/11 Hycult Biotech) and rat anti-mouse C5a (R&D Systems), rabbit anti-C5aR (Proteintech Group), mouse anti-C3aR (clone D12, Santa Cruz Biotechnology) and Alexa Fluor 647 anti-mouse CD31 (Biolegend, red fluorescence). As a loading control, we used monoclonal mouse anti- $\beta$ -actin (AC-15, Sigma Aldrich) or HRP-labeled goat anti-mouse IgG (Fc fragment, Jackson ImmunoResearch) in the case of serum. In case of Western blots, primary antibodies were detected with HRP-labeled goat anti-rat (Sigma-Aldrich), HRP goat anti-rabbit (Abcam), HRP-labeled donkey anti-goat (Abcam). In case of immunofluorescence, we used Cy2-labelled donkey anti-rat or rat anti-mouse antibodies (The Jackson Laboratory) (green) to detect primary antibodies. DAPI (Sigma-Aldrich) was used to stain nuclei (blue).

#### **Antibodies for Western blotting and immunofluorescence**

Activated complement was detected using rat monoclonal anti-mouse C3b/iC3b/C3c mAb (clone 2/11, Hycult Biotech) that specifically recognizes C3 cleavage products but not inactive C3 (Mastellos et al., 2004). Furthermore, we used monoclonal rat anti-mouse C1q (clone JL-1 Hycult Biotech), polyclonal goat anti-mouse MBL-C (Santa Cruz Biotechnology), polyclonal rabbit anti-mouse Factor B (Thermo Fisher Scientific), polyclonal rabbit anti-mouse C3 (Hycult Biotech), monoclonal rat anti-mouse C3a mAb (clone 3/11 Hycult Biotech) and monoclonal rat anti-mouse C5a mAb (clone 295103 R&D Systems), polyclonal rabbit anti-C5aR (Proteintech Group), polyclonal mouse anti-C3aR (Santa Cruz Biotechnology) and Alexa Fluor 647 anti-mouse CD31 (Biolegend, red fluorescence).

The proteins that belong to the JAK/STAT and NF $\kappa$ B pathways and their phosphorylated forms were analyzed using polyclonal rabbit anti-mouse Stat-1 (Cell Signaling), polyclonal rabbit anti-mouse P-Stat-1 (pY701) (Cell Signaling), polyclonal rabbit anti-mouse Stat-2 (Upstate), rabbit anti-mouse P-Stat-2 (pY690) (Santa Cruz Biotech), monoclonal rabbit anti-mouse Stat-3 (D3Z2G Cell Signaling), polyclonal rabbit anti-mouse P-Stat-3 (pY705) (Cell Signaling), polyclonal rabbit anti-mouse Jak1 (Santa Cruz Biotech), polyclonal rabbit anti-mouse P-Jak1 (pY1022/1023) (Cell Signaling), monoclonal mouse anti-mouse I $\kappa$ B $\alpha$  (clone 112B2 Cell Signaling) and monoclonal mouse anti-mouse P-I $\kappa$ B $\alpha$  (5A5 Cell Signaling).

As a loading control, we used monoclonal mouse anti- $\beta$ -actin (AC-15, Sigma Aldrich) or HRP-labeled goat anti-mouse IgG (Fc fragment, Jackson ImmunoResearch) and polyclonal rabbit anti-mouse transferrin (Thermo Scientific) in the case of serum. In case of Western blots, primary antibodies were detected with HRP-labeled goat anti-rat (Sigma-Aldrich), HRP goat anti-rabbit (Abcam), HRP-labeled donkey anti-goat (Abcam). Cy2-labelled donkey anti-rat or rat anti-mouse



antibodies (The Jackson Laboratory) (green) were used to detect primary antibodies. DAPI (Sigma-Aldrich) was used to stain nuclei (blue).

### **Electron Microscopy (EM)**

For EM, tissue samples at different time points from two spots of two mice were fixed in Karnovsky's fixative. Postfixation was done with 1% Osmiumtetroxide in 0,1 M cacodylatebuffer, after washing tissues were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria) followed by ethanol dehydration series. Finally the samples were embedded in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, U.S.A), sectioned and stained with Ultrastain 2 (Leica, Vienna, Austria) and analyzed with a CM10 electron microscope (FEI, Eindhoven, the Netherlands).

### **Sample preparation for flowcytometry and sorting**

Tumors were harvested and cut in small pieces followed by digestion using 1.5 mg/mL Collagenase IV (Roche) plus 100 µg/mL DNase I (Sigma-Aldrich) in media for 1 h at 37°C. Samples were filtered through a 0.45 µm cell strainer, centrifuged and resuspended in FACS buffer (PBS containing 2% FCS, 0,05% NaN<sub>3</sub>, 20 mM EDTA) or PBS, depending on the staining panels. Cells were stained with antibodies to the following markers: CD45.2 (Clone: 104, Biolegend), MHCII (Clone: M5/114.15.2, Biolegend), CD70 (Clone: FR70, eBioscience), CD86 (Clone: GL-1, Biolegend), CD11c (Clone: N418, eBioscience), CD4 (Clone: RM4-5, Biolegend), CD8β (Clone: 53-5.8, Biolegend) (Clone: YTS156.7.7, Biolegend), TCRβ (Clone: H57-597, BD Biosciences), IFNγ (Clone: XMG1.2, Biolegend), FoxP3 (Clone: FJK-16s, eBioscience), LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, Invitrogen), LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Molecular Probes, Invitrogen). Samples were measured using a CyAn ADP9 analyzer (Beckman Coulter), sorting was performed using a FACS Aria II (BD Bioscience). Data were analyzed using FlowJo software (Tree Star Inc.).

### **Human Samples**

Samples were transferred in Trizol reagent and processed according to the manufacturer's instructions for RNA (for qPCR) and protein (for Western blot) isolation. Briefly, after homogenizing the sample with the FastPrep24 homogenizer we added chloroform to separate the homogenate into a clear upper aqueous layer (containing RNA), and a red lower layer (containing the DNA and proteins). RNA was precipitated from the aqueous layer with isopropanol. DNA was precipitated with ethanol and proteins were precipitated from the phenol-ethanol supernatant by isopropanol. The precipitated RNA or proteins were washed with ethanol to remove impurities, and then dissolved in RNase-free water or 300 µL of water with 1% SDS, respectively. As only RNA samples with a RIN > 3 were used for qPCR, we excluded one sample (RIN = 2.10) from further analysis. qPCR was performed using commercially available TaqMan primers (Applied Biosystems). Results were normalized to *Gadph* and displayed as relative expression.

Isolated proteins were quantified using a Nanodrop ND-1000 spectrophotometer and loaded on 12% polyacrylamide gels. We used anti-human C3 antibody (Thermo Scientific) and monoclonal human anti- $\beta$ -actin (Sigma-Aldrich). Blots were evaluated using a Fusion-FX7 SPECTRA machine and Bio1D software (VILBER LOURMAT, Eberhardzell, Germany).

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# Radiotherapy increases the functionality of lymphatic vessels without modifying their structure

Laura Surace<sup>1</sup>, Steven Proulx<sup>2</sup>, Amanda Lund<sup>3</sup>, Melody Swartz<sup>3</sup>, Michael Detmar<sup>2</sup>, Maries van den Broek<sup>1</sup>.

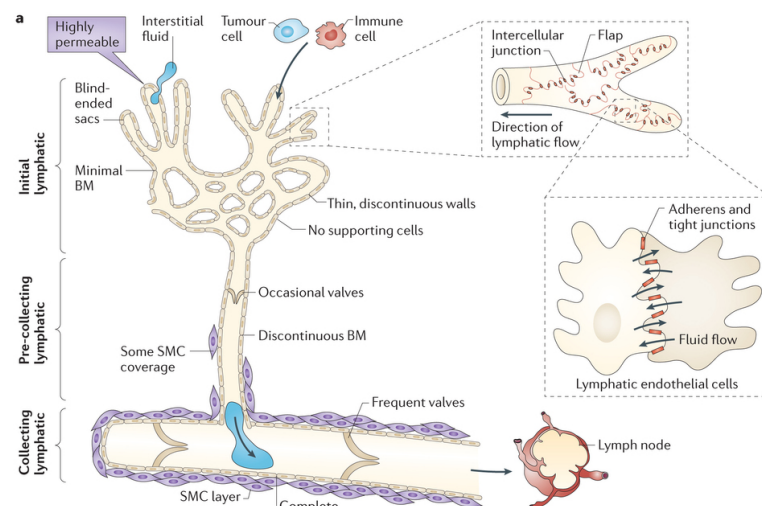
<sup>1</sup>Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland

<sup>2</sup>Institute of Pharmaceutical Science, Federal Institute of Technology Zurich (ETHZ), Zurich, Switzerland

<sup>3</sup>Institute of Bioengineering (IBI), School of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

## Introduction and Aims

The lymphatic system is a network of vessels that is generated during embryogenesis after blood vasculature is formed. Differentiated lymphatic endothelial cells (LECs) exit blood vessels and form the lymphatic sacs, an initial structure from which lymphatic vessels of peripheral organs develop (Stanczuk et al., 2015). Lymphatic capillaries in tissues are blind-ended structures that are composed of a continuous layer of loose LECs surrounded by elastic fiber. In contrast to blood vessels, lymph vessels do not contain pericytes. This conformation renders lymphatic vessels highly permeable to large macromolecules, pathogens and migrating cells (**Figure 1**).



**Figure 1. Structural organization of the lymphatic system. a)** Lymphatic vessel subtypes. The structure of the LECs in the initial lymphatics is shown in details in the two pull-out boxes (Stacker et al., 2014).

Interstitial fluid (also called lymph) enters the lymphatic vessels from the extracellular space within tissues and is transported through skeletal muscle contraction and respiratory movement. During this process, lymph is transported through the lymph nodes, is collected by larger collecting vessels finally reaching the thoracic duct from where it reaches the blood (Alitalo, 2011).

The lymphatic system transports the protein-rich interstitial fluid back to the bloodstream and facilitates the absorption of dietary fat. Besides this, the lymphatic system is a crucial component of the immune system and includes secondary lymphatic organs such as lymph nodes, tonsils, Peyer's patches and spleen. LECs regulate the trafficking of leukocytes to and from the lymph node (LN) (Pham et al., 2010). Small and large antigens collected from tissues reach the draining lymph node (DLN) through the afferent lymphatic vessels and can be taken up by antigen presenting cells (APCs). In particular, dendritic cells are specialized APCs that have a dual role in inducing adaptive immune response to foreign antigens in the presence of costimulatory signals (priming) and in maintaining T cell tolerance to self (Steinman and Banchereau, 2007).

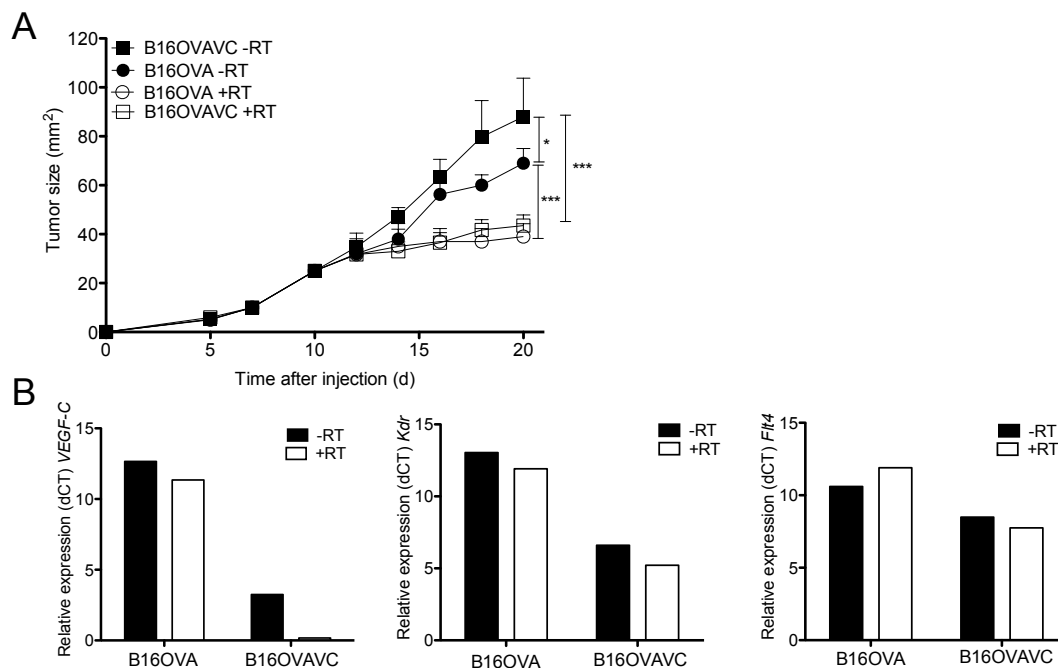
Inflammation is associated with remodeling of blood and lymphatic vessels (Halin and Detmar, 2008). For example, solid tumors structurally and functionally modulate the lymphatic system through the production of VEGF-C. The VEGFC-VEGFR3 axis is considered the most important driving factor for tumor-associated lymphangiogenesis (Tammela and Alitalo, 2010). Although lymphatic vessels were originally thought to be a passive feature in antigen transportation and metastatic tumor spread, recent evidence demonstrated that LECs are active players in both processes. It was shown that LECs take up tumor antigens and present them on MHC-I molecules to CD8<sup>+</sup> T cells. This resulted in T cell tolerance due to the lack of co-stimulatory molecules and the expression of PD-L1 on the surface of LECs (Lund et al., 2012). Many tumors have been reported to disseminate first through the lymphatic system. In human malignant melanoma, increased lymphatic density in the periphery correlated with metastasis (Shields et al., 2004). Tumor associated draining lymph nodes but not naïve lymph nodes are lymphangiogenic and with an immunosuppressive environment (Shields et al., 2010). They are shaped by the tumor in order to be an “escape route” for metastatic cells (Preynat-Seauve et al., 2007; Stacker et al., 2014).

Radiotherapy, a widely used treatment for cancer patients, has been reported to impact on the number of tumor-associated lymphatic vessels (Avraham et al., 2010). In addition, antigen presentation in the draining lymph node was improved by local irradiation of the tumor and correlated with an increased number of tumor-infiltrating CD45.2<sup>+</sup> cells (Lugade et al., 2005). However, the impact of local radiation on the structure and functionality of lymphatic vessels is still unknown. We therefore investigated the effect of radiotherapy on tumor-associated lymphatic vessels.

## Results

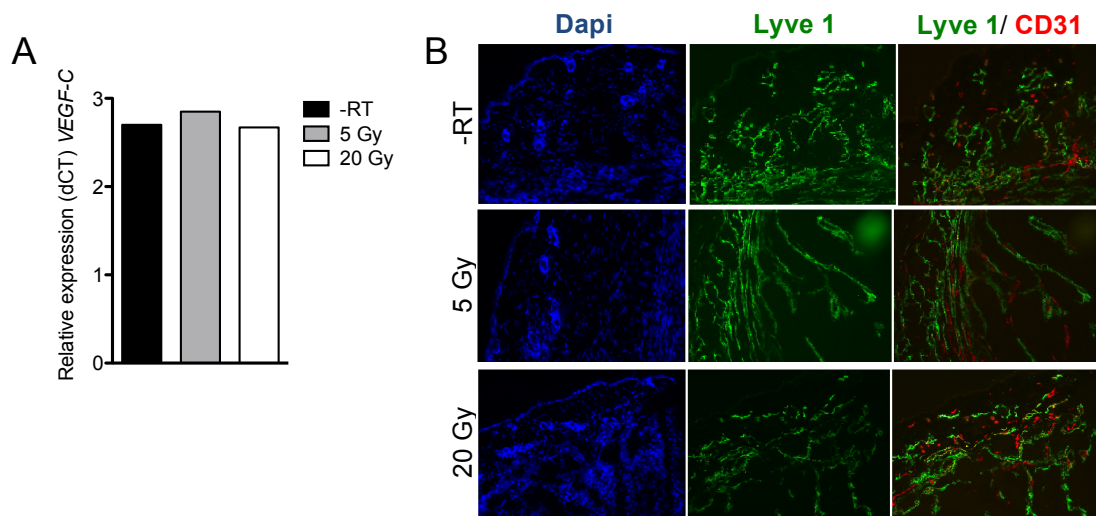
Overexpression of VEGF-C causes lymphangiogenesis and tumor progression (Skobe et al., 2001). To analyze whether the amount of lymph vessels has an effect on the efficacy of radiotherapy, we injected C57BL/6 mice s.c. with B16F10-OVA (B16-OVA) and B16F10-OVA-VEGFC (B16-

OVA-VC) cells. VEGF-C-expressing tumors contained an increased amount of peritumoral and intratumoral lymphatic vessels (data not shown). B16-OVA-VC grew significantly faster than B16-OVA, in agreement with earlier data (Skobe et al., 2001). Nevertheless, tumors responded equally well to radiotherapy applied as a single dose of 20 Gy (**Figure 1A**), suggesting that the number of tumor-associated lymph vessels does not influence susceptibility to radiotherapy. We quantified *Vegfc*, *Kdr* (VEGF receptor 2) and *Flt4* (VEGF receptor 3) by quantitative real-time PCR (qPCR) in the tumors at the end point of the experiment. As expected, the amount of *Vegfc* transcripts was elevated in B16-OVA-VC compared to B16-OVA tumors, and radiotherapy did not change this (**Figure 1B, left panel**). The amount of *Kdr* and *Flt4* were similar in both tumors and did not change upon radiotherapy (**Figure 1B, middle and right panels**).



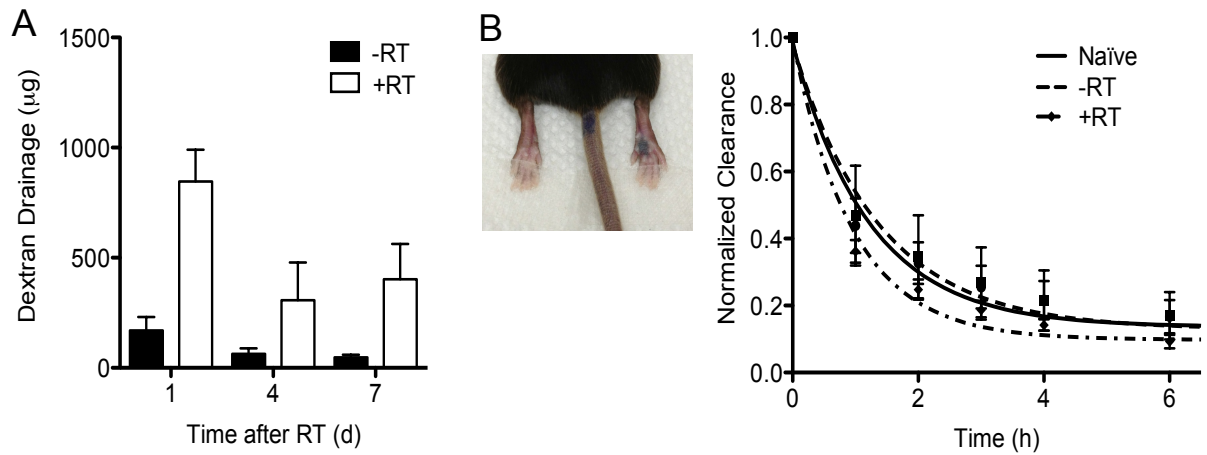
**Figure 1. Radiotherapy efficacy is not dependent on the amount of lymphatic vessels.** C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F-OVA-VC or B16F-OVA cells and were irradiated with a single dose of 20 Gy on d 12, or left untreated. **A)** Tumor growth curves. Data are shown as mean  $\pm$  SD.  $*p < 0.05$   $***p < 0.0005$  by two-way analysis of variance (ANOVA) with the Bonferroni correction **B)** Relative expression of *Vegf-c*, *Kdr*, *Flt4* transcripts in the tumor at end point. (n = 5 mice per group).

To analyze the immediate effect of different doses of radiotherapy on tumor-associated lymphatic vessels we injected B16-OVA-VC cells, irradiated established tumors with a single dose of 5 or 20 Gy and analyzed the amount of *Vegfc* transcripts by qPCR and the density of lymphatic vessels by immunofluorescence in the tumor 24 h later. Neither *Vegfc* transcripts (**Figure 2A**) nor the density of lymphatic vessels (**Figure 2B**) changed after radiotherapy, suggesting that lymphatic vessels are relatively radio-insensitive.



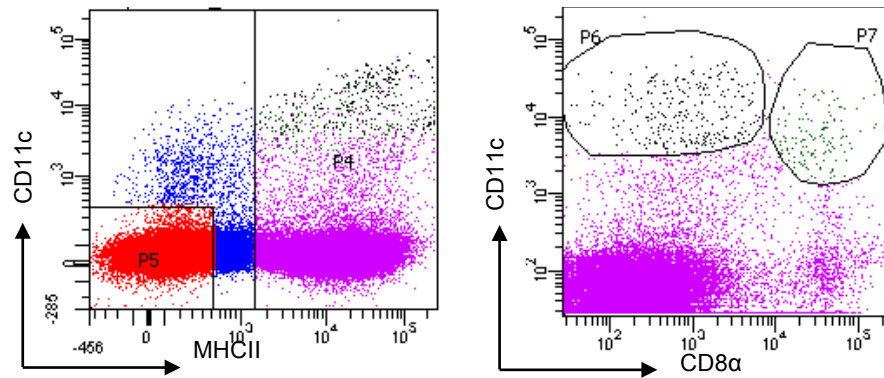
**Figure 2. Lymphatic vessels are not radiosensitive.** C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16-OVA-VC cells and were irradiated with a single dose of 20 or 5 Gy on d 12, or left untreated. Tumors were harvested 24 h after the treatment. A) Relative expression of *Vegfc* transcripts. B) Immunofluorescence untreated and irradiated tumors. Sections were stained with DAPI (left column); Lyve-1 antibody for lymphatic vessels (green fluorescence, middle column) and merged image of Lyve-1 and CD31 antibody for blood vessels (red fluorescence, right column). (n = 5 mice per group)

Tumors drive lymphangiogenesis, which occurs intratumorally, peritumorally and in the DLN. Whereas lymphatic vessels in the tumor are collapsed and not functional, peritumoral vessels are functional and associated with metastasis (Padera et al., 2002). We measured the function of lymphatic vessels function by injecting rhodamine dextran into B16-OVA tumors as described (Lund et al., 2012) or by intravital microscopy (Proulx et al., 2010). We used B16-OVA cell line instead of B16-OVA-VC to have a more physiological model. B16-OVA-VC tumors have an enormous amount of lymphatic vessels compared to B16-OVA ones (Lund et al., 2012). Using two independent methods, we observed that the function of tumor-associated lymphatic vessels was increased in irradiated compared to untreated tumors (**Figure 3A and 3B**), but additional analyses must be performed to further clarify impact of radiotherapy on tumor-associated lymphatic vessels. We could conclude that radiotherapy does not affect the structure of tumor-associated lymphatics but only the function.



**Figure 3. Radiotherapy results in increased function of tumor-associated lymphatic vessels.** A) C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16-OVA cells and received a single dose of 20 Gy on d 12, or were left untreated. Rhodamine dextran was injected intratumorally 30 minutes before harvesting tumors and draining lymph nodes at the indicated time points. (n = 5 mice per group) B) Two  $\times 10^5$  B16-OVA cells in 50  $\mu$ l PBS were injected in the footpad of C57BL/6 mice. Twelve days later, footpads were irradiated with a single dose of 20 Gy on d 12, or left untreated. LP-ICG (liposomal formulation of indocyanine green) was intradermally injected at the border of the tumor and images were acquired at the indicated time points. (n = 2 mice per group)

It was shown that cross-presentation by tumor infiltrating DCs increases upon radiotherapy in a type-I-IFN dependent manner (Burnette et al., 2011), but data on cross-presentation in the tumor-draining lymph nodes are not available. Since our preliminary data show that radiotherapy results in increased lymphatic flow, we investigated whether radiotherapy influences the amount of cross-presented tumor antigens in the draining lymph node. Therefore, we injected C57BL/6 mice with B16-OVA or B16-OVA-VC cells and irradiated tumors 12 d later with 20 Gy or left them untreated. Two days after radiotherapy, DLN were harvested, pooled per experimental group and stained for sorting. Sorting was performed according to the gating strategy in **Figure 4**. We isolated CD8 $\alpha^+$  DC (P7, CD45.2 $^+$  CD11c $^+$  MHCII $^{\text{high}}$  CD8 $\alpha^+$ ), CD8 $\alpha^-$  DC (P6, CD45.2 $^+$  CD11c $^+$  MHCII $^{\text{high}}$  CD8 $\alpha^-$ ) and a negative fraction (P5, CD45.2 $^+$  CD11c $^-$  MHCII $^-$  CD8 $\alpha^-$ ).



**Figure 4. Sorting of dendritic cells from DLN of untreated and irradiated tumors.** C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16-OVA-VC or B16-OVA cells and received a single dose of 20 Gy 12 d later. Draining lymph nodes were harvested 2 days later and pooled for sorting analysis. The plot on the left shows P5 population (red)  $CD11c^- MHCII^-$  (gated on  $CD45.2^+$  live singlets). The plot on the right shows P6 (black)  $CD8\alpha^+ CD11c^+ MHCII^{high}$  and P7 (green)  $CD8\alpha^- CD11c^+ MHCII^{high}$  (both previously gated on P4).

We repeated the experiment in order to reach the required amount of cells ( $\approx 10^7$ ) for the proteomic analysis (LC-MS-MS) performed by Prof. Anthony Purcell (Monash University, Melbourne) as previously reported (Gerlach et al., 2011). The total amount of cells collected from the different sorting for the three populations is reported in the table (**Table 1**). We did not find any difference in the amount of cells recovered from untreated and irradiated tumors.

	B16-OVA -RT	B16-OVA +RT
neg fraction	$2.06 \times 10^7$	$3.4 \times 10^7$
$CD8\alpha^- DC$	$2.18 \times 10^5$	$2.57 \times 10^5$
$CD8\alpha^+ DC$	$8.64 \times 10^4$	$8.57 \times 10^4$
	B16-OVA-VC - RT	B16-OVA-VC +RT
neg fraction	$3.53 \times 10^7$	$3.03 \times 10^7$
$CD8\alpha^- DC$	$2.01 \times 10^5$	$1.89 \times 10^5$
$CD8\alpha^+ DC$	$1.08 \times 10^4$	$9.19 \times 10^4$

**Table 1. Total amount of cells for the different sorted populations.** The total number of cells for the different populations from DLN of mice injected with B16-OVA and B16-OVA-VC irradiated or not (+/- RT) are listed in the second and third columns. Negative fraction (neg) indicates  $CD11c^- MHCII^-$  cells (gated on  $CD45.2^+$  live singlets),  $CD8\alpha^+ DC$  indicates ( $CD8\alpha^+ CD11c^+ MHCII^{high}$ ) and  $CD8\alpha^- DC$  ( $CD8\alpha^- CD11c^+ MHCII^{high}$ ).



We aimed to quantify the amount of ovalbumin-derived SIINFEKL peptide presented by H-2K<sup>b</sup> by mass spectrometry (Croft et al., 2013), however, the amount of SIINFEKL was under the detection limit in all samples (data not shown). To better address the question additional experiments are needed including a kinetic analysis of the samples described above.

## Discussion

Lymphatic vessels have been reported to have a controversial role in cancer development. They are fundamental to develop a tumor-specific immune response because they transport tumor antigens and dendritic cells to secondary lymphoid organs where T cells are primed. On the other hand it has been proven that LECs and factors released by the tumor, can render T cells ineffective by creating a suppressive environment (Munn and Mellor, 2006). Moreover, lymphatic vessels together with blood vessels are the only possible route for tumor cells to metastasize; in fact regional lymph nodes are the first sites of metastasis in many cancers. Metastases in the lymph node are considered a negative prognostic factor for cancer patients (Sleeman and Thiele, 2009) and are used in the clinics to define the stage of the tumor (Leong et al., 2006). Nevertheless extensive studies on tumor-associated lymphatic vessels have been performed; their role in the development of tumor-specific immunity, tumor growth and the formation of metastases is still unclear.

Radiotherapy modulates the tumor microenvironment and promotes inflammation (Thompson and Maity, 2014). We embarked to investigate whether radiotherapy impacts on the structure and/or the function of tumor-associated lymphatic vessels. Since VEGF-C is the factor driving lymphangiogenesis and it has also been correlated with increased formation of metastases (Pepper et al., 2000), we therefore analyzed the amount of VEGF-C transcripts upon radiotherapy. We could not find any difference in expression levels comparing irradiated tumors to untreated ones. At the functional level, we could show an enhanced drainage upon radiotherapy but no changes in the structure of tumor-associated lymphatic vessels. One possible explanation for our observations could be that the increase in drainage is a direct consequence of the radiotherapy-mediated inflammation. In fact, it has been published that inflammatory processes cause expansion of the lymphatic network and increase in the drainage (Kim et al., 2012). Changes in the function of tumor-associated lymphatic vessels, as an increase in the drainage, might have consequences on the type of immune response developed in the DLN. As previously reported for other models, continuous exposure to the antigen can lead to the promotion of tolerance (Vignali et al., 2008). Characterizing the dynamics of antigen transportation to the DLN upon radiotherapy could be interesting to better understand the development of radiotherapy-mediated tumor-specific immunity. For example, recent studies have highlighted a new role of lymphatic endothelial cells on T-cell fate and function. It has been shown that LECs, that lack co-stimulatory molecules and express PD-L1 (Tewalt et al., 2012), can actively scavenge antigens and present them on MHC class I molecules driving immune tolerance (Lund et al., 2012). LECs can also directly dampen DC maturation (Podgrabska et al., 2009). It would be interesting to repeat the analysis of changes in

the antigen load by mass spectrometry in the DLN upon radiotherapy and couple it with an extensive study of the DLN environment and immune response.

In conclusion, it could be highly relevant to better characterize lymphatic vessel modification upon irradiation in order to clarify if the can be exploited improve the current status of the therapy.

## Material and Methods

### Cell lines and mice

C57BL/6 (C57BL/6JOLA<sup>Hsd</sup>) mice were purchased from the Harlan (Rijswijk, The Netherlands). All experiments were performed with age- and sex-matched mice in accordance with the guidelines of the Swiss federal and cantonal laws on animal protection.

B16-OVA (B16F10 stably transfected to express chicken ovalbumin as neo-antigen) and B16-OVA-VEGF-C (B16-OVA stably transfected to express VEGF-C) were provided by Melody Swartz, EPFL, Lausanne, Switzerland). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) supplemented with 10% fetal calf serum,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10 mM sodium pyruvate 2 mM L-glutamine and antibiotics.

### *In vivo* experiments

Two  $\times 10^5$  tumor cells were injected s.c. on the left or right flank of mice in 100  $\mu$ l of a 1:1 mix of PBS and Matrigel™ Basement Membrane Matrix (BD Biosciences). For footpad injection  $2 \times 10^5$  cells were injected in a volume of 50  $\mu$ l of PBS.

Local radiotherapy with a single dose of 20 or 5 Gy was performed at indicated time points using a Xstrahl 200 kV X-ray unit at 1 Gy/min as described (Gupta et al., 2012). Prior to radiotherapy, mice were anaesthetized by i.p. injection of 50 mg/kg ketamine and 10 mg/kg xylazine. Tumors were measured with a caliper every 2-3 days in two dimensions (length and width).

### Quantitative real-time PCR (qPCR)

Excised tumors were collected at the indicated time points in 1 ml Trizol Reagent (Life Technologies) per 0.2 g of tissue. Samples were homogenized with the FastPrep24 homogenizer (MP Bio, France) for 2 cycles of 30 seconds according to the manufacturer's instructions and RNA was isolated according to the Trizol protocol. The concentration and the purity of the extracted RNA were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with a RIN (RNA integrity number) > 3 were used for subsequent qPCR. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. qPCR was performed using commercially available TaqMan primers for *Vegfc*, *Kdr* and *Flt4* (Applied Biosystems). Reactions were run on the RotorGene Cyclor (Qiagen). Results were normalized to *Gadph* and displayed as relative expression.

### Immunofluorescence

Irradiated and untreated tumors were collected in OCT at the indicated time points (Tissue-Tek Optimal Cutting Temperature Compound, Sakura), gently frozen in liquid nitrogen and stored at -80°C for 24 h. Samples were then left for 30' at -20°C and processed into 7-µm thick sections using a microtome at -20°C. Cryosections were blocked for 1 h at room temperature in a blocking solution (PBS containing 10% FCS and 0.1% TritonX), washed with PBS and incubated overnight at 4°C with primary antibodies diluted in blocking solution. Sections were washed with PBS and incubated with secondary antibodies diluted in blocking solution for 1 h at room temperature. Slides were mounted in Mowiol anti-fade solution (Sigma-Aldrich). Sections were photographed using a Leica DMI6000 inverted microscope (Leica microsystem, Wetzlar, Germany). Sections were evaluated using ImageJ software.

Blood vessels were detected using Alexa Fluor 647 anti-mouse CD31 antibody (Biolegend, red fluorescence), lymphatic vessels were detected using rabbit polyclonal anti-mouse Lyve-1 antibody; DAPI (Sigma-Aldrich) was used to stain nuclei. Cy2-labelled donkey anti-rabbit (The Jackson Laboratory) (green) were used to detect rabbit-anti-mouse Lyve-1 primary antibody.

### **Function of lymphatic vessels**

Twenty µl of rhodamine dextran were injected into untreated and irradiated tumors on one flank of mice. After 30 minutes, tumors and tumor draining lymph nodes were harvested. LNs were homogenized and fluorescence was quantified by plate reader.

For the live imaging of lymphatic vessels function, mice were anesthetized with 2% isoflurane and placed inside IVIS Spectrum (Xenogen, Caliper Life Sciences). The imaging parameters were set as reported in (Proulx et al., 2010). Five µl of the liposomal (LP) ICG (15 µmol/L) was injected at the border of the primary tumor in the footpad and images were acquired at the indicated time points. The liposomal formulation of indocyanine green (LP-ICG) is described to be specifically taken up by lymphatic vessels (Proulx et al., 2010). For image analysis, Living Image Software (Caliper Life Sciences) was used. For assessments of flow through the inguinal lymph node, the data were analyzed as described before (Proulx et al., 2010).

### **Sorting of tumor-associated dendritic cells**

Tumors were harvested and cut in small pieces followed by digestion using 1.5 mg/mL Collagenase IV (Roche) plus 100 µg/mL DNase I (Sigma-Aldrich) in media for 1 h at 37°C. Samples were filtered through a 0.45 µm cell strainer, centrifuged and resuspended in PBS. Cells were stained with antibodies to the following markers: CD45.2 (Clone: 104, Biolegend), MHCII (Clone: M5/114.15.2, Biolegend), CD11c (Clone: N418, eBioscience), CD8α (Clone: 53-6.7 Biolegend), LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, Invitrogen).

Samples were sorted using a FACS Aria II (BD Bioscience). Data were analyzed using FlowJo software (Tree Star Inc.).

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## Discussion

*Calor* (heat), *dolor* (pain), *tumor* (swelling) and *rubor* (redness) are the cardinal signs of inflammation identified by the Roman encyclopaedist Aulus Cornelius Celsus in AD 30. Inflammation is a physiological response to perturbation such as infection or tissue damage that aims to restore the steady state as soon as possible. Because inflammation is a potent and in principle destructive response, it is tightly regulated and such controlled inflammatory response is beneficial to the host. In contrast, chronic inflammation seems uncoupled from the initial event including infection or tissue repair and is associated with an imbalance of physiological responses. Chronic inflammation is detrimental and causes a wide array of pathologies such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, type 2 diabetes, Alzheimer's disease, cardiovascular disease, multiple sclerosis and cancer (Balkwill and Mantovani, 2001; Medzhitov, 2008).

Acute inflammation is triggered by infection or injury and results in coordinated recruitment of leukocytes to the affected site. The response to pathogens is better characterized than that to injury and starts with the recognition of the microbe by tissue-resident macrophages and mast cells, resulting in local production of pro-inflammatory mediators including vasoactive amines and peptides, lipids, enzymes, chemokines, cytokines and fragments of complement components such as anaphylatoxins (C3a, C5a) (Medzhitov, 2008). Upon elimination of the infectious agent, inflammation resolves and tissue repair is initiated (Serhan and Savill, 2005).

Failure to clear the pathogen or restore homeostasis results in chronic inflammation with changed characteristics to avoid collateral damage to the host. For example, the infiltrate contains more macrophages and lymphocytes than neutrophils, tertiary lymphoid structures may be formed and anti-inflammatory and immunosuppressive factors such as IL-10 are produced. In addition, processes associated with tissue repair and destruction occur simultaneously (Drayton et al., 2006; Medzhitov, 2008).

Already in the 19<sup>th</sup> century, Rudolf Virchow observed infiltrating leukocytes in the tumor and suggested a possible link between inflammation and cancer (Virchow, 1881). Nowadays inflammation is considered a hallmark of cancer (Hanahan and Weinberg, 2011). Apparently at odds with this theory, Coley and Fehleisen independently observed that erysipelas – a severe infection of the skin with *Streptococcus pyogenes* – resulted in cure of some cancer patients and suggested that inflammation leads to tumor regression (Coley, 1891; Modlin, 2012).

This apparent paradox can be explained by the nature of the inflammatory response: Acute inflammation supports immunity and presumably tumor control, whereas chronic inflammation is associated with immunosuppression, tumor progression (Coussens et al., 2013), increased proliferation rates resulting in high mutation rates of some cells (Grivennikov et al., 2010;

Mantovani et al., 2008), angiogenesis and endothelial-to-mesenchymal transition (EMT) (Thiery, 2002), suggesting that inflammation not only plays a role in the initial phase of tumor development, but also in metastasis formation.

The strong correlation between chronic inflammation and cancer provides a rationale for using anti-inflammatory drugs in cancer prevention and therapy. For example, prophylactic use of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) reduced the incidence of colorectal, lung and esophageal cancer (Baron and Sandler, 2000). In addition, chronic low-dose aspirin intake because of a heart condition prevented the development of metastasis in several common cancers during and after trials (Rothwell et al., 2011; Rothwell et al., 2012). Because aspirin and NSAIDs interfere with many pathways including inflammation, clotting and others, the mechanistic basis for the beneficial action of such drugs in the context of cancer is currently not completely understood.

Radiotherapy is a standard treatment for cancer patients and may represent a practical example of how acute and chronic inflammation differently impact on the tumor and its microenvironment. Radiotherapy induces DNA damage and ultimately cell death to which rapidly dividing cells, such as tumor cells, are more sensitive. Radiation-sensitivity of tumor cells is further enhanced by frequently occurring mutations in DNA-repair pathways (Begg et al., 2011).

In the last years, it has become clear that radiotherapy modulates innate and adaptive immunity and that such responses are an integral part of the clinical response to radiotherapy (Apetoh et al., 2007; Burnette et al., 2011; Gupta et al., 2012; Sharma et al., 2013). Lee and colleagues used B16 melanoma to show that single high-dose (20 Gy) or fractionated (3x 15 Gy) radiotherapy similarly delay tumor growth and depend on CD8<sup>+</sup> T cells in the tumor (Lee et al., 2009). In the same model, radiotherapy induces increased expression of VCAM-1 on tumor endothelium favoring T cell infiltration (Lugade et al., 2005). The enhanced infiltration of CD8<sup>+</sup> T cells into the tumor upon radiotherapy also depends on an increased expression of CXCL16 (Matsumura et al., 2008). Our lab demonstrated that the clinical efficacy of single high-dose radiotherapy depends on DCs maturation and activation of tumor-specific CD8<sup>+</sup> T cells (Gupta et al., 2012). The secretion of high mobility group box 1 (HMGB-1) by dying tumor cells has been suggested as upstream mechanism for DC activation (Apetoh et al., 2007). Moreover, *in vitro* and *in vivo* models showed radiotherapy-mediated upregulation of MHC class I (Reits et al., 2006; Sharma et al., 2011) and tumor necrosis factor-alpha (TNF- $\alpha$ ) (Hallahan et al., 1989). Our lab showed that radiotherapy upregulates several immune effector molecules and concomitantly downregulates immune suppressor molecules using paired biopsies from sarcoma patients (Sharma et al., 2013). Recently, it has been demonstrated in mice and humans that combination of two checkpoint inhibitors, anti-CTLA-4 and anti PD-1/PD-L1, with radiotherapy give rise to a better immunity against the tumor, compared to radiotherapy alone (Twyman-Saint Victor et al., 2015).

Systemic immunity against cancer is crucial to control of metastatic lesions and there is evidence that the combination of radiotherapy with immune stimulation may result in systemic tumor-

specific immunity and control of distant, i.e. non-irradiated, lesions (abscopal effect) (Postow et al., 2012; Verbrugge et al., 2014). All together, these finding in pre-clinical and clinical models demonstrate that radiotherapy elicits an immune response against the tumor that might be additionally potentiated by the combination with immunotherapy.

Usually, patients are treated with external-beam fractionated radiotherapy given as daily low doses (1.5-2 Gy) over multiple weeks. Because this method allowed normal tissue to repair, it was established in the clinics at a time when radiation could not yet be targeted precisely to the tumor (Martin and Gaya, 2010). Advances in radiotherapy planning software, image guidance and treatment delivery have led to the development of hypo-fractionated radiotherapy, during which radiation is given as high doses (8-20 Gy) in fewer fractions. Clinical trials comparing the two radiation schemes showed that hypo-fractionated radiotherapy is characterized by a very accurate delivery of the dose, thus reducing the damage to the healthy tissue in the proximity of the tumor and consequently secondary symptoms. Moreover, this treatment has lower treatment cost and is not time-consuming.

Currently, data comparing the clinical efficacy of both protocols are not yet available (Vaidya et al., 2010). The impact of hyper- and hypofractionated radiotherapy on inflammatory and immune responses has not been systematically studied, but we expect that hyper- but not hypofractionated radiotherapy will lead to a state of chronic inflammation. This may explain the apparent contradiction between our work and a recent paper by Elvington et al. (Elvington et al., 2014): Elvington et al. showed that inhibition of complement improved the efficacy of radiotherapy, whereas we found that local, radiotherapy-induced production of the anaphylatoxins C3a and C5a is essential to stimulation of protective immunity and efficacy of radiotherapy (Surace et al., 2015b). Complement is a potent pro-inflammatory cascade that is activated in response to disturbance of homeostasis (Corrales et al., 2012; Markiewski et al., 2008; Nunez-Cruz et al., 2012) and chronic complement activation may contribute to pathological conditions associated with chronic inflammation (Ricklin and Lambris, 2013). Whereas we used a single dose of 20 Gy, Elvington et al. used multiple low-dose irradiations on consecutive days, resulting in transient and chronic complement activation, respectively. Blockade of complement activation altogether by dexamethasone decreased the efficacy of single, high-dose radiotherapy (Surace et al., 2015b). These findings suggest that induction of an acute inflammatory response is an upstream event determining the therapeutic efficacy of radiotherapy. In stark contrast, hyper-fractionated radiotherapy delivered as daily fractions of 1.5 Gy or 7 Gy on five consecutive days resulted in chronic complement activation and increased infiltration of regulatory T cells in the tumor, but no protective tumor-specific immune response. We thus think that radiotherapy given in fewer fractions of higher dose with breaks in will result in (repeated) acute inflammatory responses that promote radiotherapy-mediated, tumor-specific immunity. Instead, conventional, hyper-fractionated radiotherapy may induce a state of immunosuppressive, chronic inflammation.

In conclusion, we propose that eliciting acute inflammation in the tumor microenvironment boosts the tumor-specific immune response. Consequently, radiotherapy should be given as a single dose or as repeated doses with intervals (radiation holidays), which results in peaks of beneficial, acute inflammation. Such a schedule may result in a superior therapeutic responses compared to the conventional schedule of daily treatment (Surace et al., 2015a).

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Virchow, R. (1881). An Address on the Value of Pathological Experiments. *British medical journal* 2, 198-203.

# Curriculum Vitae Laura Alba Maria Surace

**Laura Alba Maria Surace**

Date of birth     September, 4<sup>th</sup> 1988  
Nationality       Italian

## EDUCATION

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- November 2011- to September 2015**     University of Zurich, Switzerland  
Institute of Experimental Immunology  
PhD student in the laboratory of Experimental Oncology  
Supervisor: Prof. Maries van den Broek  
Thesis: “Radiotherapy supports tumor specific immunity by acute inflammation”
- October 2009 – July 2011**                     University of Pavia, Italy,  
Laurea Magistralis in Molecular Biology and Genetics  
Master Degree in Molecular Biology and Genetics  
Thesis: “Analysis of the molecular interactions between host protein Hck and viral protein HIV-1 infectivity viral factor Vif”
- October 2006 – July 2009**                     University of Pavia, Italy,  
Bachelor Degree in Biomolecular Biology  
Thesis: “Description of the site-specific mutagenesis assay through PCR at the C-terminal of the accessory viral protein HIV-1 Vif in order to characterize the functional domains involved in Reverse Transcriptase interaction”
- September 2001 – July 2006**                     Liceo Scientifico “A. Volta”, Reggio Calabria, Italy  
Diploma of high-school education in science

## PUBLICATIONS

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- Surace L, Guckenberger M, van den Broek M. Radiation holidays stimulate tumor immunity. *Oncotarget*. 2015 Jun 30;6(18):15716-7.
- Surace L, Lysenko V, Fontana AO, Cecconi V, Janssen H, Bicvic A, Okoniewski M, Pruschy M, Dummer R, Neefjes J, Knuth A, Gupta A, van den Broek M. Complement is a central mediator of radiotherapy-induced tumor-specific immunity and clinical response. *Immunity*. 2015 Apr 21;42(4):767-77.
- Surace L, Nicole Scheifinger, Anurag Gupta, Maries van den Broek. Radiotherapy supports tumor-specific immunity by acute inflammation. *Oncoimmunology*. 2015

## LANGUAGES

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**Italian** (mother tongue); **English** (advanced); **Spanish** (very good command); **German** (basic)

## MEETINGS AND AWARDS

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- Italian Life Sciences Federation (FISV) congress” - Riva del Garda (TN) Italy, 23-25 September 2009. “Identification of independent domains important for the physical and



functional interaction of the HIV-1 auxiliary protein *vif* with the HIV-1 reverse transcriptase”. A. Kataropoulou et al.

- “European society for Virology - 4<sup>th</sup> European congress of virology” - Villa erbe congress centre – Cernobbio, Como Lake, ITALY April 7-11, 2010.
- “ENII Spring School on Advanced Immunology – Ralph Steinman Memorial” (April 2012) and **prize for the best poster “Outstanding poster award”**
- “SIICA-SGAI Joint Workshop Targeting Dendritic Cells for Immunity and Tolerance”. November 2012. Poster presentation.
- “11th Charles Rodolphe Brupbacher Symposium Breakthroughs in Cancer Research and Therapy” – January 2013 - University Hospital Zurich. Poster presentation.
- “5<sup>th</sup> Cancer Network Retreat” April 2013 – Grindelwald. Poster presentation and selection for a talk. **Poster prize.**
- “12th Day of Clinical Research meeting” April 2013 – University Hospital Zürich. Poster presentation and selection for a talk.
- “7<sup>th</sup> International Conference on Complement Therapeutics” (June 2014) – Greece. **Talk and travel award.**
- “12th Charles Rodolphe Brupbacher Symposium Breakthroughs in Cancer Research and Therapy” – January 2015 - University Hospital Zurich. Poster presentation.

## Acknowledgments

I would like to start thanking my supervisor Prof. Maries van den Broek, who believed in me since the very first moment. She gave me the enormous chance to work on a completely new topic and in such nice environment. I learnt a lot thanks to her constant supervision, the time she spent sharing her scientific knowledge and her valuable advise that helped and supported me during these years.

Many thanks to my PhD thesis committee: Maries, Prof. Burkhard Becher, Prof. Sabine Werner, Prof. Martin Pruschy. They gave many important inputs during the development of the project. “Collaboration” is a very important word in science, so I would like to thank all the people that were involved in my project. Prof. Martin Pruschy (University Hospital Zurich) gave us access to the irradiation unit and helped establishing the technique. Dr. Andrea Fontana (Prof. Pruschy’s lab) was always precise and available and most importantly a good friend. Prof. Sjaak Neefjes and Hans Janssen (Netherland Cancer Institute, Amsterdam) performed electron microscopy, analyzed the data and gave important inputs during the preparation of the manuscript. Prof. Melody Swartz (University of Chicago) provided the cell line for the project and participated, together with Dr. Amanda Lund, on the project about radiotherapy and lymphatic vessels. Prof. Reinhard Dummer provided patient biopsies. Prof. Jovan Pavlovic (Institute of Medical Virology, University of Zurich) and Prof. James Di Santo (Department of Immunology, Institute Pasteur, Paris) provided some useful antibodies. Dr. Michal Okoniewski performed all the bioinformatic analyses. Dr. Stefano Ferrari (Institute of Molecular Cancer Research, University of Zurich), Prof. Mark Robert Nicolls (Department of Medicine, Stanford Medicine), and Prof. John Lambris (Department of Pathology, University of Pennsylvania) provided scientific support and suggestions to improve the manuscript. Many thanks to the staff of the animal facilities for expert animal care.

I would like also to thank Prof. Alexander Knuth for the support at the beginning of my project, the PhD cancer program for the precious work they have done during these years and the people from the Institute of Experimental Immunology, our new home, who are all very talented and nice. I would like to thank the present and the former members of the lab for creating a very nice and stimulating environment. In particular Virginia and Giovanni, for being great colleagues and friends. Oscarito Salgado and Paulino Tallon de Lara, los mejores profesor de Espanol y compañeros. Antonela and Veronika for working very hard on the project. Dr. Alessandra Curioni for the valuable scientific inputs and for spreading her highly contagious enthusiasm. Many thanks to Karina Silina for the useful discussions, technical support and the good time spent together and to Claudia Matter for helping me to get started with my experiments.

These years in Zurich would have been completely different without the new friends I have met here, thanks to all of you. I would also like to thank all those friends who never stopped to support me and stood by my side also when we were distant.

Many thanks to my family (too many to mention one by one) for the faith they always had in me. Having so many supporters makes life smoother. I would like to dedicate my years and achievements in Zurich to my mother and my father. The efforts they made during these years and their constant love helped me through all difficult and happy moments.

Finally, I would like to thank to my boyfriend Oscar. He made everything easier supporting all my choices. He gave me the strength to hold on and get here, being always my “safe place”. All I achieved is half his.